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Evidence now indicates that the interaction between HER-2 and HER-3 receptor kinases leads to the constitutive activation of HER-2/HER-3 heterodimers in breast cancer cells with HER-2 gene amplification, and HER-2/HER-3 then potently activates multiple signal transduction pathways that are involved in mitogenesis. This also suggests that inhibition of the interaction between HER-2 and HER-3 may be an effective and unique strategy for blocking the actions of HER-2 in human breast cancer cells. Therefore, we constructed various retroviral expression vectors that code for a dominant negative form of HER-3 which inhibits the function of HER-2/HER-3. We used these dominant negative HER-3 vectors in experiments to determine the effectiveness of dominant negative HER-3 for blocking HER-2/HER-3 activation and growth in culture and *in vivo* for different breast cancer cell lines with HER-2 gene amplification. Dominant negative HER-3 was able to inhibit the activation of HER-2/HER-3 and the proliferation of cells stimulated by heregulin (the ligand for HER-2/HER-3), as well as their growth factor-independent (i.e. autonomous) proliferation in culture, anchorage-independent growth in soft agarose, and tumor growth *in vivo*. Our work also suggested that stoichiometric considerations involving different mutant/wild-type receptor levels in cells may also be important for blocking HER-2/HER-3.

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## INTRODUCTION

The HER-2 (*neu*/*erb*B-2) gene encodes a 185 kDa protein tyrosine kinase that is highly homologous to the epidermal growth factor (EGF) receptor (EGFR/HER-1/*erb*B- 1), HER-3 (*erb*B-3), and HER-4 (*erb*B-4) (1-3), which together, comprise the type 1 family of receptor tyrosine kinases (4, 5). These receptors differ in their ligand specificities (4), and while HER-1 binds several ligands closely related to EGF, HER-3 and HER-4 are the receptors for a number of different isoforms of *neu* differentiation factor/heregulin (HRG) (6-8). While no direct ligand for HER-2 has yet been cloned, it is now clear that HER-2 is capable of heterodimerization with HER-1 (9, 10), HER-3 (11, 12), or HER-4 (8), and these HER-2-containing heterodimers form the highest affinity binding sites for their respective ligands (10, 11). HER-2 is amplified in 28% of primary breast carcinomas *in vivo* (13), and another 10% overexpress HER-2 without amplification of the gene (14-16). HER-2 gene amplification, concordant with high-level overexpression, is associated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (13, 14, 17-19). Experimentally elevated HER-2 gene expression in various cell lines induces tumorigenesis in nude mice (20-23), and the potent oncogenic potential of HER-2 is generally thought to be due to its ability to constitutively activate key signaling pathways involved in the regulation of cell growth (24). Although HER-2 was originally discovered as the *neu* transmembrane-mutated form of the gene in rat neuroblastoma cells (25), the HER-2 gene found in human breast cancer cells has never shown such mutations (26). The level of tyrosine-phosphorylated HER-2 in primary breast cancer *in vivo* always shows a direct correspondence with HER-2 overexpression (27). While the wild-type HER-2 protein possess constitutive tyrosine kinase activity when overexpressed in cell lines in the absence of any identifiable ligand (20-23, 28, 29), the HER-2 tyrosine kinase domain is also constitutively active in EGFR-HER-2 chimeric receptors in the absence of EGF (28, 29). Therefore, the overexpression of wild-type HER-2 alone is sufficient to constitutively activate its tyrosine kinase function. Additionally, heterodimeric interactions are now known to occur between the different HER proteins, including HER-1 and HER-2 (9, 10), HER-2 and HER-3 (11, 12), HER-2 and HER-4 (8), and HER-1 and HER-3 (30, 31). Our own work and that of others has now shown that heterodimer interactions between HER-2 and HER-3 are constitutively active in breast cancer cells with HER-2 gene amplification (32-35), and co-transfection of HER-3 with HER-2 greatly augments the transforming capability of HER-2 in genetically engineered cell lines (33). Therefore, we are particularly interested in how the cooperative effects of HER-2/HER-3 heterodimers activate key mitogenic signaling pathways that facilitate cell growth. Our own work and that of others has also now shown that the constitutive activation of HER-2/HER-3 in breast cancer cells is associated with the constitutive activation of phosphatidylinositol (PI) 3-kinase and mitogen-activated protein (MAP)-kinase (32-37). One strategy that has been used successfully to block the function of other receptor tyrosine kinases employs dominant negative expression vectors in which a region coding for the cytoplasmic domain of the receptor is almost completely removed. While the truncated receptor still contains the extracellular and transmembrane domains and can dimerize within the cell membrane, it lacks tyrosine kinase activity and inhibits the signal transduction docking function of mutant/wild-type heterodimers (Fig. 1A). This strategy has been used effectively to block EGFR (38), platelet-derived growth factor receptor (39), and fibroblast growth factor receptor (40) in biochemistry studies. Recently, a dominant negative HER-2 vector was also used successfully to block HER-2 function in normal mouse mammary gland

development (41). The use of such dominant negative HER-2 vectors has not yet been reported to block HER-2 in cancer cells with HER-2 gene amplification. This is probably due to the stoichiometric probems associated with the inability to generate mutant/wild-type levels high enough for effective inhibition in human breast cancer cells with HER-2 gene amplification. However, the fact that HER-3 is not highly overexpressed in these cells, and that activated HER/HER-3 has a particularly high-affinity interaction (42-44), suggests that dominant negative HER-3 may be especially effective in blocking HER-2/HER-3 function. Therefore, we have introduced a dominant negative form of HER-3 into cells in an attempt to block the activation of HER-2/HER-3 in breast cancer cells.

## BODY

In this final report, we describe the work performed throughout the entire period of the Idea Award, including data attained during the suggested no-cost extension of the grant this last year. This report has also been written in a manner to conform with the additional suggestions made by previous reviewers as well. In particular, the description of experiments has now been expanded to include explicit reference to the specific statement of work as well as the periods of time in which the work was performed and completed. A summary follows that specifically details the fulfillment of all the objectives and specific tasks outlined within the original grant proposal.

### *Completion of Work Involving the 21MT-1 Breast Carcinoma Cell Line*

The completion of work involving the 21MT-1-derived cell lines was performed during the first year of the grant period. These cell lines had been developed prior to the start of the grant and were used to attain the data included in the original grant proposal. We routinely use H16N-2 normal breast epithelial cells and 21MT-1 breast carcinoma cells for our studies because they were derived from the same patient and can be grown under precisely defined serum-free conditions in culture. These cells express EGFR, HER-2 and HER-3 and are highly responsive to the growth-stimulatory effects of EGF, insulin and HRG in serum-free culture (34, 45). This well defined system was then used to study growth factor-independent (i.e. autonomous) proliferation and responses to exogenous growth factors in a manner that is not yet possible for other cell lines derived in medium containing high levels of serum and that are routinely propagated under such undefined conditions in culture. Therefore, this serum-free system was well suited to study receptor activation and signaling in a manner which allowed us to distinguish constitutive from externally mediated growth factor responses in culture during prolonged periods of serum deprivation (34, 45).

The original approach for developing the dominant negative HER-3 expression vector employed the full-length human HER-3 cDNA from which a 2.2 kb fragment was removed and inserted into a bicistronic retroviral expression vector as previously described (46; see attachment). By using the pBK-CMV phagemid expression vector (Stratagene) as an intermediate, we cloned the dominant negative HER-3 fragment into a bicistronic retroviral expression vector originally derived from pLNCX by using flanking restriction sites located within the extensive polylinker region of pBK-CMV. The 2.2 kb fragment of HER-3 was first cloned into the polylinker sites of the pBK-CMV vector (46), which also introduced an in-frame stop codon downstream of the point of ligation. The resulting vector, pBK-CMV-dn3, was then used directly in experiments in which the vector was transfected into both the H16N-2 normal cell line and the 21MT-1 metastatic tumor cell line with HER-2 gene amplification. However, the transfection of standard expression vectors into these and other cell lines did not show very high transfection efficiencies or coordinate co-expression of antibiotic resistance genes with ectopic marker genes in host cell lines in culture. The efficiency of expression of ectopic marker genes was dramatically improved by using retroviral expression vectors infected into cell lines, and was greatest when using bicistronic vectors in which in the antibiotic resistance gene and the gene-of-interest are driven by a single promoter. Therefore, we constructed a bicistronic retroviral

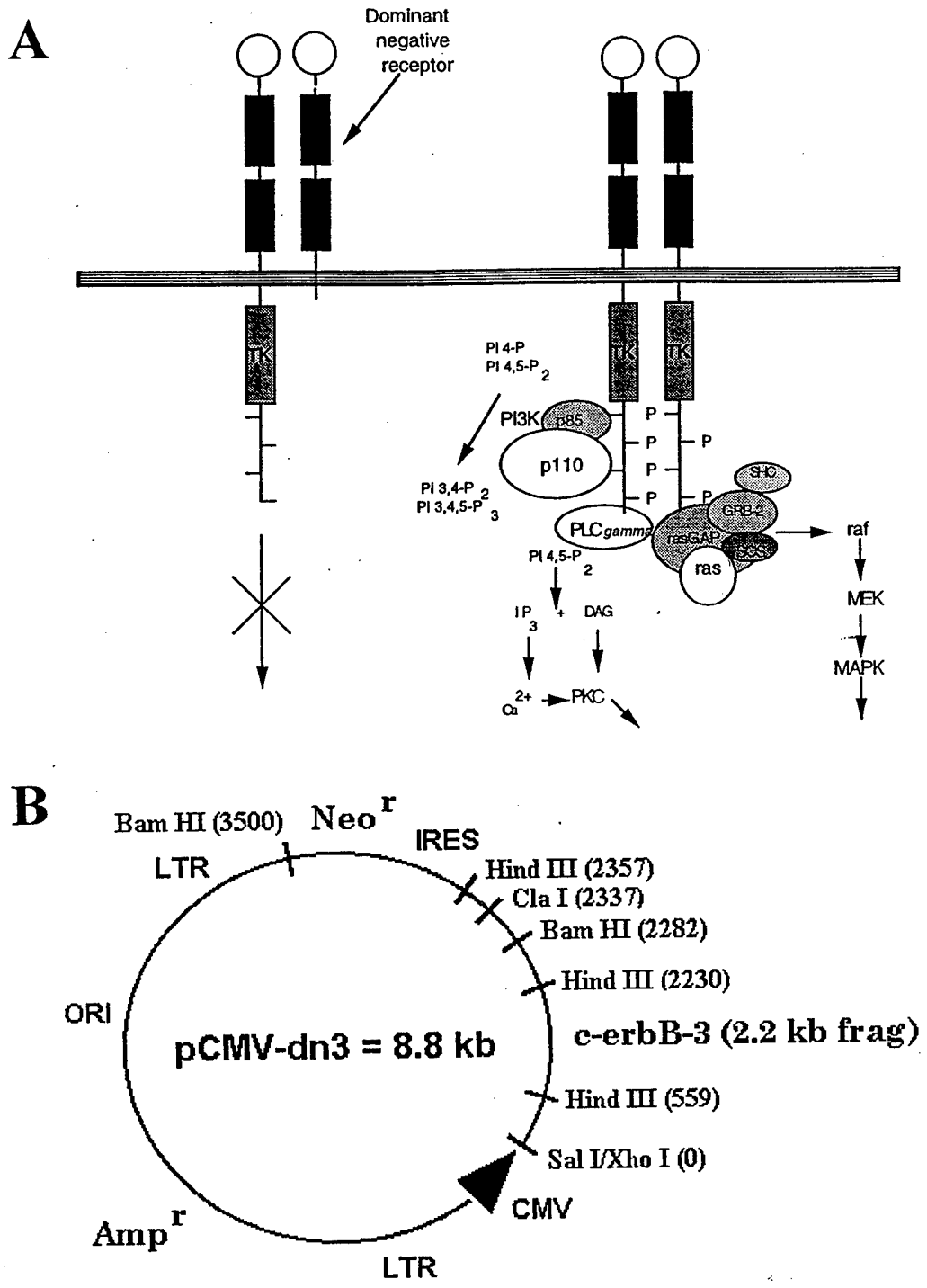



Fig. 1. *Bicistronic Retroviral Expression Vector for Dominant Negative HER-3.* (A) Diagram showing the strategy for blocking wild-type receptor tyrosine kinases with dominant negative (i.e. truncated) receptors. (B) The pCMV-dn3 bicistronic retroviral expression vector was constructed containing a dominant negative form of the HER-3 gene in which most of the cytoplasmic domain of HER-3 was removed. In addition, this vector contains an internal ribosome-binding site (IRES) between the HER-3 fragment and the neo$^r$ gene located downstream, which together, form a single transcription unit when expressed in mammalian cells. The expression of ectopic genes by a bicistronic transcript then allows for the 100% coordinate co-expression of the ectopic gene with antibiotic resistance in infected cells selected on antibiotic (46; see attachment).

expression vector that resulted in 100% coordinate co-expression of the neo$^r$ gene with the Lac Z marker gene, thus eliminating the occurrence of false positive clones when ectopic genes was infected into cells using this bicistronic vector (46). The dominant negative form of HER-3 was then successfully cloned into the bicistronic retroviral expression vector to generate pCMV-dn3 (Fig. 1B). H16N-2 and 21MT-1 cells were then infected with the pCMV control vector or pCMV-dn3, selected on G418, and then used to screen for the expression of dominant negative HER-3, and to determine its effect on HER-2/HER-3 activation, signaling and growth responses in culture (46). The expression of pCMV-dn3 was found to strongly inhibit both the constitutive tyrosine phosphorylation of HER-2/HER-3 in the 21MT-1 cells as well as the HRG-induced activation of HER-2/HER-3 in both the H16N-2 and 21MT-1 cells in culture (46). Anchorage-dependent monolayer growth assays performed under serum-free conditions also showed that dominant negative HER-3 strongly inhibited both the autonomous proliferation of the 21MT-1 cells in the complete absence of exogenous growth factors, as well as the HRG-induced proliferation of both H16N-2 and 21MT-1 cells in culture (46). In contrast, dominant negative HER-3 had no visible effect on the insulin/EGF-induced proliferation of either H16N-2 or 21MT-1 cells in culture (46). Therefore, the effects of pCMV-dn3 showed selectivity by preferentially inhibiting growth responses involving HER-2/HER-3, such as those that are constitutively activated by HER-2 or that are induced by HRG. The anchorage-independent growth of 21MT-1 cells in soft agarose was also potently inhibited by dominant negative HER-3, further showing that dominant negative HER-3 was able to block the transformed growth of these cells in culture (46).

The fact that dominant negative HER-3 had no apparent effect on the insulin/EGF-induced proliferation of either H16N-2 or 21MT-1 cells in culture suggested that dominant negative HER-3 preferentially inhibited HER-2/HER-3-mediated growth responses over those induced by exogenous EGF. This result had especially important significance, because it also indicated that the effects of dominant negative HER-3 preferentially inhibited proliferation induced by HRG, or that is a result of constitutive activation of HER-2/HER-3 in breast cancer cells. This also indicated that EGFR/HER-3 is not as effectively inhibited by dominant negative HER-3 as is HER-2/HER-3 at a given level of dominant negative HER-3 gene expression, or that the interaction between EGFR and HER-3 is not required for EGF-induced proliferation. Apparently, it was this preferential inhibition of HER-2/HER-3 that then allowed us to use a constitutive promoter to express dominant negative HER-3 in cells that were still able to proliferate in response to EGF. While EGFR and HER-3 do interact in these and other cell lines (47), clearly the EGFR/HER-3 heterodimer interaction is weak compared to the HER-2/HER-3 heterodimer interaction (42-44). To further investigate the preferential effects of dominant negative HER-3 on HER-2/HER-3-mediated growth responses in the 2MT-1 cells, we also compared the levels of receptor activation in cells stimulated by either HRG or EGF (Fig. 2). While dominant negative HER-3 inhibited the constitutive tyrosine phosphorylation of HER-2/HER-3 as well as that seen with HRG stimulation, the level of EGF-induced tyrosine phosphorylation of EGFR-containing dimers was not apparently affected by dominant negative HER-3 in the 21MT-1 cells (Fig. 2). These results are consistent with the hypothesis that EGFR-containing dimers are not as effectively inhibited by dominant negative HER-3 as are HER-2/HER-3 heterodimers at a given level of dominant negative HER-3 gene expression.

*In vivo* studies were also performed using the 21MT-1-derived cell lines for injections into immunodeficient mice as originally described in the grant proposal. However, the 21MT-1 cells

were not tumorigenic in our studies and thus would not allow us to test the effects of dominant negative HER-3 *in vivo* using 21MT-1 cells. This is apparently a common problem for a significant number of malignant and metastatic breast carcinoma cell lines (48), and earlier studies also suggested some difficulty in using 21MT-1 cells for tumor studies at later passages (49). Additional experiments were performed during the first year of the grant period comparing injections of 21MT-1 cells in both Nu/Nu and Nu/Nu CD-1 strains of nude mice, as well as in SCID mice that lack both T-cell and B-cell function. However, we were not able to detect any tumors in mice injected with up to $10^7$ cells per site in any of the strains of immunodeficient mouse yet tested. Interestingly, we also noticed on further review of the literature that most previous studies employed fibroblasts genetically engineered to overexpress HER-2 for their *in vivo* experiments, rather than spontaneously derived breast carcinoma cell lines with HER-2 gene amplification. While the 21MT-1-derived cell lines were useful for the initial characterization of the effects of dominant negative HER-3 in cell culture studies, the inability of these cells to form tumors *in vivo* presents a serious limitation for their use for more extensive studies of the effects of dominant negative HER-3 on tumorigenesis.

*Using BT-474 Cells for Tumorigenesis Studies In Vivo*

During the first two years of the grant period, we also performed transplants in immunodeficient mice using the BT-474, MDA-MB-453 and SK-BR-3 breast carcinoma cells as originally outlined in the grant proposal. BT-474, MDA-MB-453 and SK-BR-3 cells have all been reported to contain HER-2 gene amplification as well as activation of HER-2/HER-3, so we had proposed to use these cell lines in addition to 21MT-1 cells. After obtaining the cell lines directly from the ATCC, experiments were conducted to measure their tumorigenicity *in vivo* as well as the level of their HER-2/HER-3 expression and receptor activation in culture. None of these cell lines have yet been found to form detectable tumors in either Nu/Nu or Nu/Nu CD-1 strains of nude mice injected with $10^7$ cells per site and left to grow for at least 2 full months. However, BT-474 cells were found to consistently form palpable tumors in SCID mice within a few weeks after injection. BT-474 tumors were dissected out and measured after 2 months from the time of injection. There was a wide range in the size of the tumors (Fig.3), but these cells did form tumors in every case. Histological characterization of these tumors was also performed (Fig. 4). However, the relatively small size of BT-474 tumors would not allow for more extensive biochemical characterization of tumors as outlined in the original grant proposal, making these parts of the grant (Task 6) not applicable. Experiments injecting MDA-MB-453 or SK-BR-3 cells in SCID mice also showed some very small tumors, but these tumors were not palpable and were detected only after autopsy more than 5 months
after the time of injection.

Analysis of HER-2 and HER-3 expression and activation was carried out using the BT-474, MDA-MB-453 and SK-BR-3 cell lines during the first two years of the grant period. All 3 of these cell lines showed very high levels of HER-2, and was highest in the BT-474 cells (data not shown). HER-3 was expressed by all 3 cell lines, and was moderately elevated in the BT-474 and MDA-MB-453 cells relative to the SK-BR-3 cells. In addition, the constitutive activation of HER-2 and HER-3 was also measured in the BT-474, MDA-MB-453 and SK-BR-3 cells. The cells were placed in serum-free medium for 48 hours prior to protein extractions so that the levels

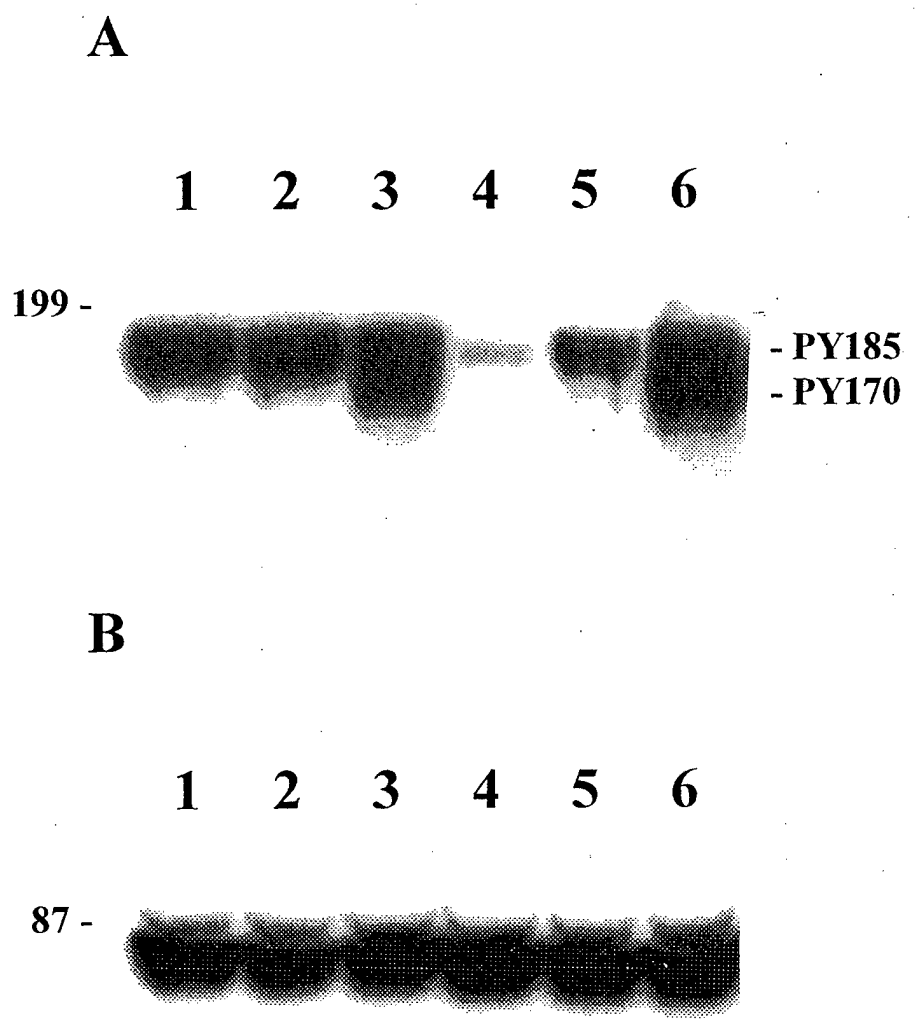



Fig. 2. *Preferential inhibition of HER-2/HER-3 tyrosine phosphorylation in 21MT-1 cells expressing dominant negative HER-3.* A) Samples containing 200 μg cell lysate protein per lane were immunoblotted with anti-phosphotyrosine antibody. B) The same blot was then reprobed with anti-p85 antibody as a control to confirm equal loading of the gel. 21MT-1 cells previously infected with either pCMV (Lanes 1-3) or pCMV-dn3 (Lanes 4-6) and selected on G418 were then deprived of growth factors for 48 hours in serum-free medium and then directly extracted (Lanes 1 and 4), or stimulated with 10 ng/ml HRG-β (Lanes 2 and 5), or stimulated with 10 ng/ml EGF (Lanes 3 and 6) for 10 minutes at 37° C before cell lysate extraction.

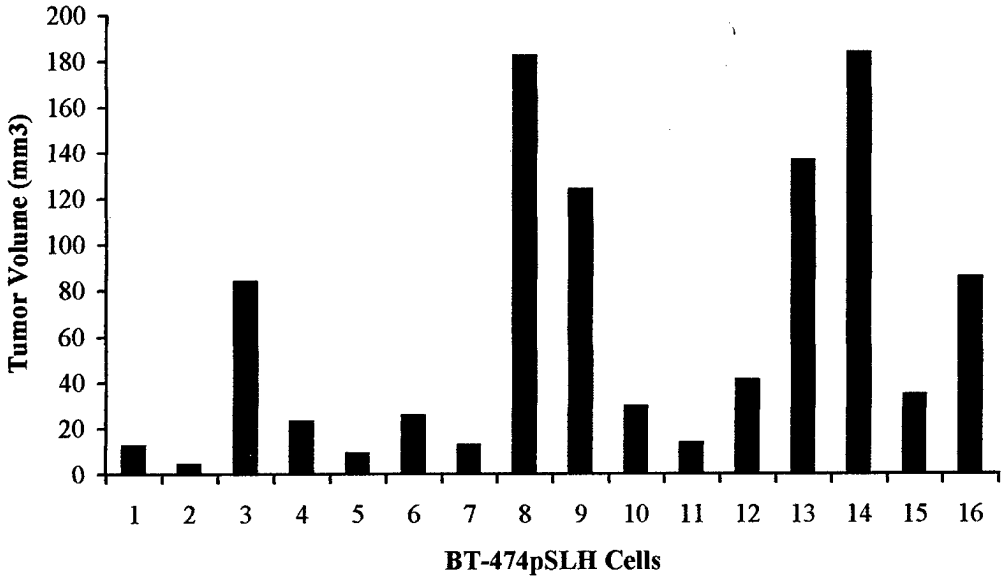



Fig. 3. *Growth of BT-474 control cell tumors in SCID mice.* Histogram showing the distribution of tumor sizes for BT-474pSLH cells grown for 2 months in female SCID mice. Cells ($10^7$) were injected s.c. in a solution containing 50% Matrigel and the tumors were removed after they had grown for 2 months *in vivo*, measured with vernier calipers, and tumor volumes (in $mm^3$) were calculated using the formula [volume = 0.52 x $(width)^2$ x (length)] according to Bergers et al., 2000 (50).

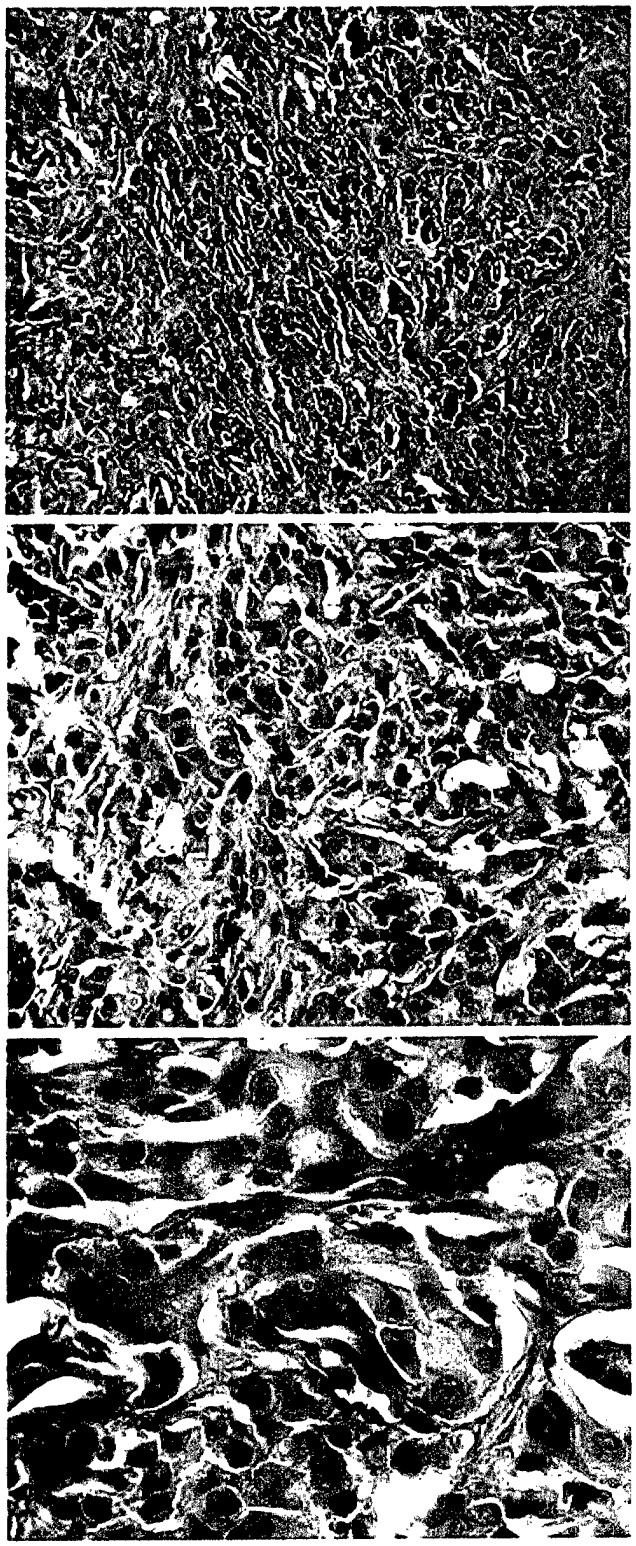


Fig. 4. *Histological analysis of BT-474 control cell tumors grown in SCID mice.* H & E staining of sections prepared from BT-474pSLH cell tumors grown for 2 months in female SCID mice. Cells ($10^7$) were injected s.c. in a solution containing 50% Matrigel and the tumors were removed after 2 months, fixed in 10% Formalin solution and processed according to standard procedures for histological analysis. Bright field microscopy of tissue sections is shown at 4X (A), 10X (B) and 20X (C) magnification.

of tyrosine-phosphorylated receptors represented the *constitutive* level of receptor activation in these cells. Immunoprecipitation/Western blot measurement of tyrosine-phosphorylated HER-2 and HER-3 also showed that both HER-2 and HER-3 were most highly tyrosine-phosphorylated in the BT-474 cells (data not shown). SK-BR-3 cells showed moderately high levels of HER-2 tyrosine-phosphorylation, but lower levels of HER-3 tyrosine-phosphorylation. MDA-MB-453 cells showed the lowest levels of HER-2 and HER-3 tyrosine-phosphorylation. Western blotting of PI 3-kinase and SHC proteins in anti-phosphotyrosine immunoprecipitates also showed that BT-474 cells had much higher levels of $p85^{PI\ 3\ kinase}$, $p46^{SHC}$ and $p52^{SHC}$ recruited by activated HER-2/HER-3 than that seen in the MDA-MB-453 and SK-BR-3 cells (data not shown).

*Construction of BT-474, MDA-MB-453 and SK-BR-3 Cells Expressing Dominant Negative HER-3*

During the first year of the grant period, we infected BT-474, MDA-MB-453 and SK-BR-3 cells with pCMV and pCMV-dn3 as outlined in the original grant proposal. Titrations were initially performed to determine the concentrations of G418 necessary to kill BT-474, MDA-MB-453 and SK-BR-3 cells within 2-3 weeks after addition of the antibiotic. These concentrations (i.e. 500 ug/ml for SK-BR-3 cells, 600 ug/ml for MDA-MB-453 cells and 700 ug/ml for BT-474 cells) were then used to select these cell lines on G418 after being infected with pCMV or pCMV-dn3. Infections of all the cell lines were first carried out using ψCRIP-derived packaging cell lines previously transfected with pCMV or pCMV-dn3 and selected on G418. However, no colonies grew out for BT-474, MDA-MB-453 and SK-BR-3 cells infected with conditioned medium prepared from ψCRIP-derived packaging cell lines. These ψCRIP packaging cells are not thought to produce very high viral titers compared to other packaging cell lines freshly selected for packaging function. Therefore, we then transfected the PA-317 packaging cell line (ATCC) with pCMV or pCMV-dn3 and selected them on 800 ug/ml G418 for a month to derive new packaging cell lines that were then used for infecting BT-474, MDA-MB-453 and SK-BR-3 cells. By using the PA-317-derived packaging cell lines, we then successfully generated viral titers sufficient for good colony formation of BT-474, MDA-MB-453 and SK-BR-3 cells infected with the pCMV control vector. Interestingly, we routinely saw a much lower number of colonies growing out for the cells infected with pCMV-dn3 under identical conditions in culture. Earlier observations were then extended during the second year of the grant period with additional experiments done in triplicate where the colonies were counted after infection and selection on G418 for a month in culture (Fig. 5). Furthermore, we also noticed that many of the colonies that started to grow out in plates infected with pCMV-dn3 were either very small (Fig. 6B) or did not continue to grow, instead showing gradual morphological deteriation and eventual cell death (data not shown). This suggested that the growth of many of the cells infected with pCMV-dn3 may be inhibited by the expression of dominant negative HER-3 during their initial selection on G418.

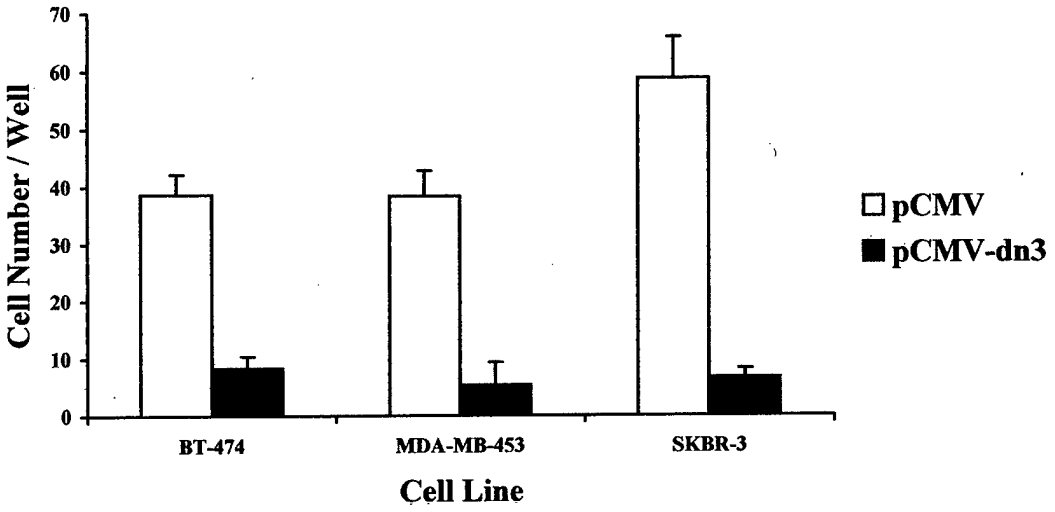



Fig. 5. *Clonal outgrowth of BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV or pCMV-dn3.* BT-474, MDA-MB-453 and SK-BR-3 cells were infected for 3 days with conditioned medium collected from PA-317 packaging cells stably transfected with pCMV or pCMV-dn3. The infected cells were then incubated with fresh medium for 2 days and selected on G418 for a month before counting colonies in 6 well plates. The mean average and standard deviation for triplicate wells is shown. All of these cell lines show a highly significant reduction in cell colony number when cells infected with pCMV-dn3 are directly compared with cells infected with pCMV ($p < 0.05$). The StatMost program (Dataxiom Software Inc) was used to calculate significance in paired t-Tests.

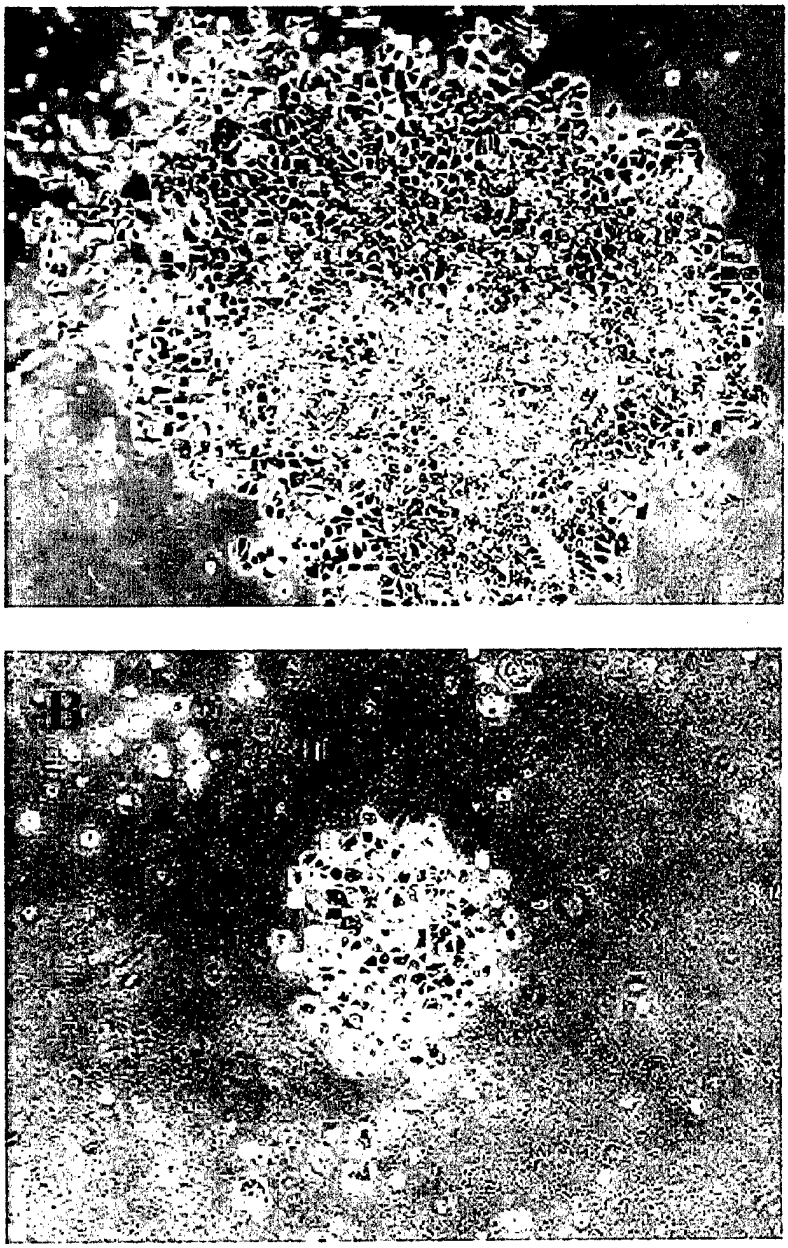



Fig. 6. *Morphology of BT-474 cell colonies infected with pCMV or pCMV-dn3*. Phase contrast microscopy of BT-474 cells infected with either pCMV (A) or pCMV-dn3 (B) and selected on G418 for a month in culture. Notice the smaller size of colonies that is routinely seen for cells infected with pCMV-dn3.

*Expression of Dominant Negative HER-3 in BT-474 Cell Populations and Cell Clones in Culture*

In order to confirm that dominant negative HER-3 was expressed in BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV-dn3, we performed anti-HER-3 immunocytochemistry on cells infected with pCMV or pCMV-dn3 after being selected on G418 and passaged as pooled colonies (i.e. mass selected) in culture. These experiments were completed during the first year of the grant period as outlined in the original grant proposal. The H105 monoclonal anti-HER-3 antibody (Neomarkers) is known to be highly specific for HER-3 and binds to an epitope within the extracellular domain of HER-3. While this antibody does not work for Western blotting, it worked well for immunocytochemistry (Neomarkers; 46). While all of the cell lines used in these studies expressed wild-type HER-3, the levels are relatively low when detected with HRP/DAB staining in cells infected with pCMV compared to cells infected with pCMV-dn3 (46). Therefore, the high levels of HER-3 measured in cells infected with pCMV-dn3 confirmed the ectopic expression of dominant negative HER-3. However, we also noticed significant heterogeneity in the levels of HER-3 staining in cell populations infected with pCMV-dn3 (see Fig. 5 in first annual report) and fewer dark staining cells after passaging in culture. Such heterogeneity in dominant negative HER-3 expression in these cells also suggested the possibility that there may be a preferential selection of cells expressing lower levels of dominant negative HER-3 if it inhibits the growth of these cells during the initial selection on G418. These mass selected BT-474, MDA-MB-453 and SK-BR-3 cell populations infected with either pCMV or pCMV-dn3 were also used for experiments as outlined in the original grant proposal to screen for the levels of HER-2, HER-3, PI 3-kinase and MAP-kinase activation. BT-474-derived cells were also tested for their tumorigenicity *in vivo* during the first two years of the grant period as outlined in the original grant proposal. These mass selected and pooled cell populations showed no significant differences in the levels of HER-2, HER-3, PI 3-kinase and MAP-kinase activation, and the BT-474 cells infected with pCMV-dn3 showed no significant reduction in tumor growth in SCID mice *in vivo* (data not shown). However, these results were difficult to interpret because this may also indicate that the mass selected and extensively passaged cell populations may not retain many cells expressing very high levels of dominant negative HER-3 if they are being selected out during extensive passaging in culture.

To further investigate the levels of expression of dominant negative HER-3 in cell colonies immediately after selection on G418, additional anti-HER-3 immunocytochemistry experiments were performed during the second year of the grant period (Fig. 7). While some of the colonies infected with pCMV-dn3 expressed very high levels of dominant negative HER-3 (Fig. 7B), including some very small dying colonies (data not shown), other cell colonies showed only moderate or low levels of staining for HER-3 (Fig 7C; upper left). Some heterogeneity of staining was also seen within individual colonies. A number of the cell clones from pCMV-dn3-infected BT-474 cell populations were also isolated and screened for their expression of dominant negative HER-3. Additionally, the acquisition of a polyclonal anti-HER-3 antiserum directed against the extracellular domain of HER-3 (Transduction Labs) then allowed us to identify the dominant negative HER-3 protein in Western blots for cell clones at early passage in culture (Fig. 8). Interestingly, some of the slowest growing clones that required a much longer time to grow out were the samples that showed the highest levels of the approximately 100 kD dominant negative HER-3 protein (Fig. 8, Lanes 2, 6, 8 and 9). In

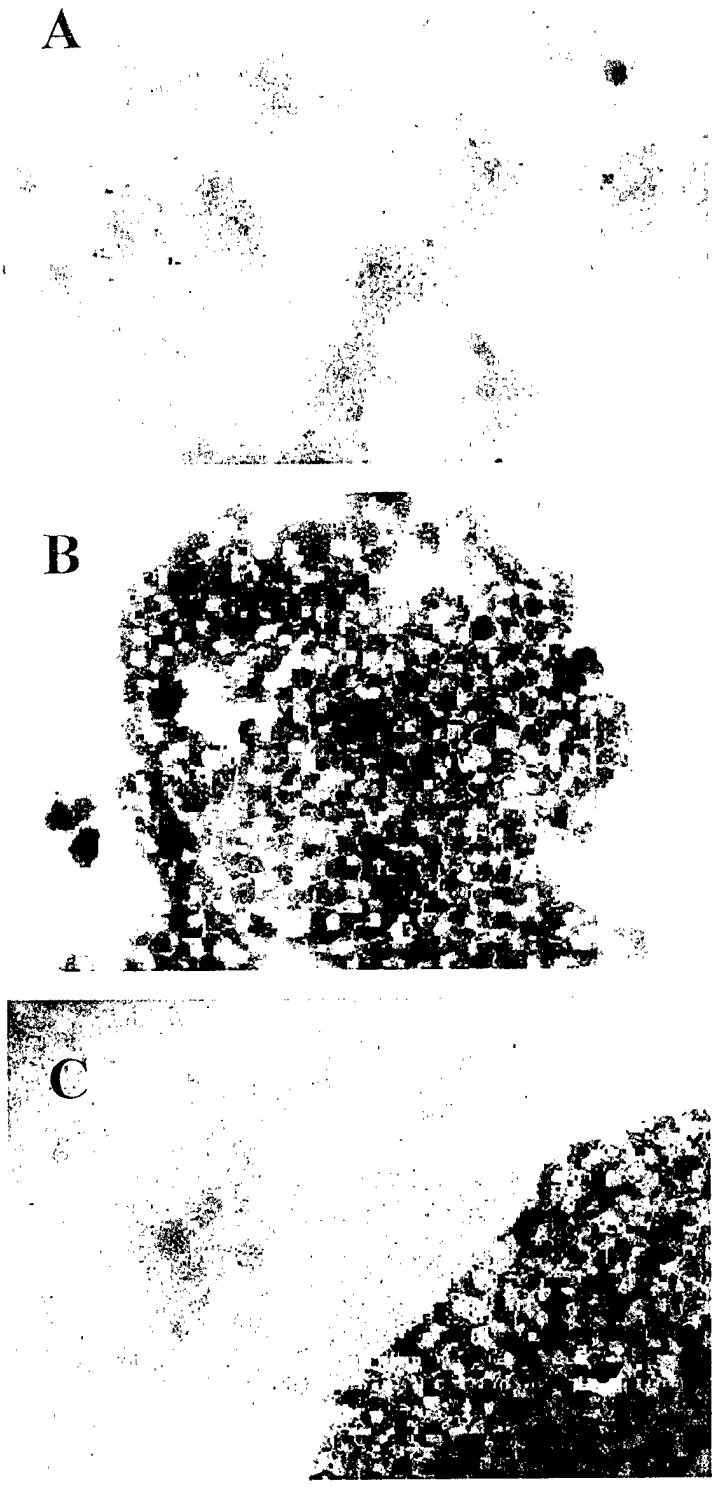



Fig. 7. *Expression of dominant negative HER-3 in BT-474 cells infected with pCMV-dn3.* Bright field microscopy of anti-HER-3 immunocytochemistry in BT-474 cells infected with either pCMV (A) or pCMV-dn3 (B and C). High levels of ectopic HER-3 was seen in many slow growing and dying (not shown) cell colonies. Also notice the heterogeneity in staining between different BT-474 cell colonies infected with pCMV-dn3 (C), where some colonies express much lower levels (C, upper left) while others express high levels (C, lower right) of dominant negative HER-3 in adjacent colonies within a well containing various cell clones shown right after being selected on G418 for a month.

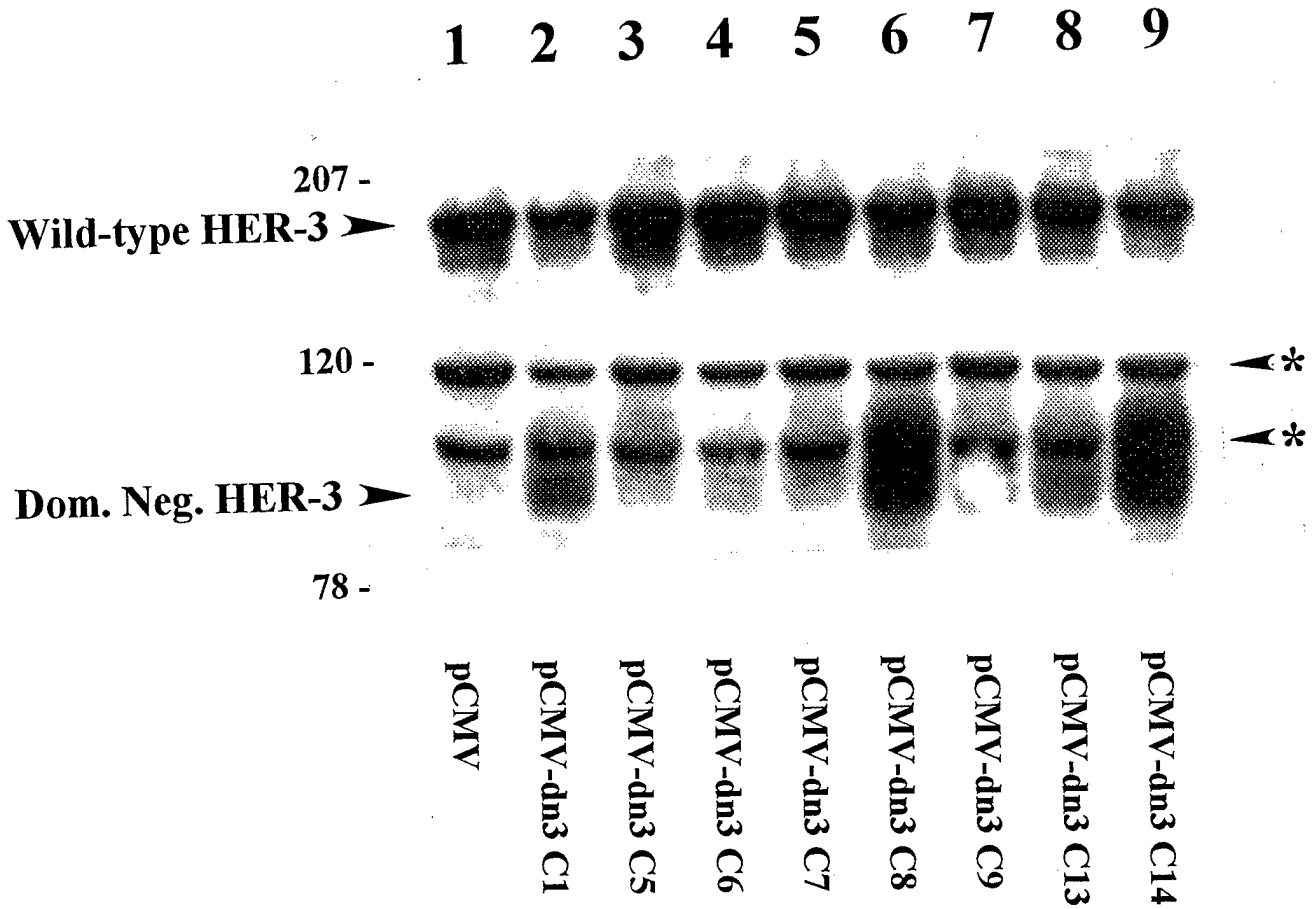



Fig. 8. *Expression of dominant negative HER-3 in BT-474 cell clones infected with pCMV-dn3.* Western blot analysis of HER-3 expression in BT-474 cells infected with pCMV (Lane 1) or different BT-474 cell clones infected with pCMV-dn3 (Lanes 2 -9). Samples containing 100 ug cell lysate protein were immunoblotted with a polyclonal anti-HER-3 antibody raised to the extracellular region of HER-3 to directly identify the approximately 100 kD dominant negative HER-3 protein in cells infected with pCMV-dn3 (the arrow points to the lower most intense region of the very broad band that is always seen for dominant negative HER-3). The clones shown in Lanes 2, 6, 8 and 9 expressed the highest levels of dominant negative HER-3. High molecular weight markers are shown (kD) on the left and non-specific bands are marked ( * ) on the right.

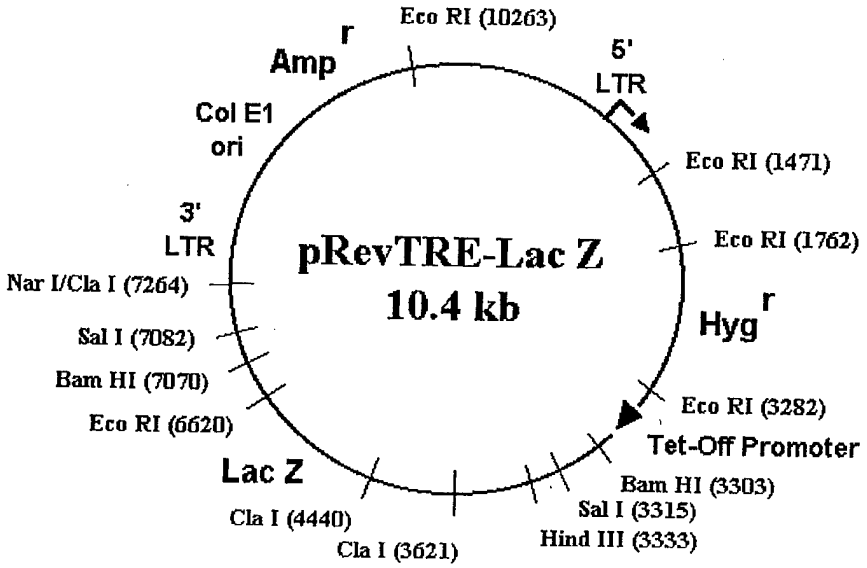
addition, the dominant negative form of HER-3 was seen as a broad band in Westerns, possibly indicative of differential glycosylation and/or other modification of the ectopic protein in genetically engineered cell lines. There may be some potential for using these BT-474-derived cell clones at early passage. However, we may also have difficulty in maintaining cell lines with stable high-level dominant negative HER-3 gene expression during extensive passaging in culture because these cells may require constitutive HER-2/HER-3 function for their propagation in culture.

*Construction of Vectors and Cell Lines for Expressing InducibleDominant Negative HER-3 Using the "Tet-Off" System*

If the highest level expressing cells are being selected out because of the inhibitory effects of the ectopic dominant negative HER-3 gene, this may necessitate the use of an inducible expression vector to study the full extent of the effects of dominant negative HER-3 in cell lines requiring HER-2/HER-3 constitutive function for propagation in culture. Therefore, we also developed retroviral expression vectors for engineering cell lines that express regulatable dominant negative HER-3 using the Tetracycline repressor ("Tet-Off") system during the second and third year of the grant period. The tetracycline-repressible pRevTRE retroviral expression vector facilitates the regulated expression of ectopic genes in mammalian cells (Clontech). So we then constructed an inducible dominant negative HER-3 vector using the pRevTRE vector. pRevTRE contains Sal I and Cla I sites that were used to insert the Sal I-Cla I dominant negative HER-3 fragment isolated from pBK-dn3 (46) into pRevTRE to generate pRevTRE-dn3 (Fig. 9B). We also constructed a control vector, pRevTRE-Lac Z, which was used to determine the levels of tetracycline repressor activity in cell lines stably infected with pRevTet-Off (which contains the tetracycline repressor and neomycin-resistance genes). pRevTRE-Lac Z was cloned by inserting the Hind III-Nar I Lac Z fragment isolated from pSV-β-Galactosidase (Promega) into the Hind III and Cla I sites (Nar I ends are also compatible with Cla I ends) in pRevTRE to generate pRevTRE-Lac Z (Fig. 9A). The proper construction of the vectors was verified with extensive restriction digest analysis (Fig. 10). The newly constructed pRevTRE-dn3 vector was then used to infect cells that had been previously infected with pRevTet-Off (using media from PA-17 cells transfected with pRevTet-Off and selected on G418), selected on G418 and screened for tetracycline repressor function. A number of cell clones infected with pRevTet-Off were isolated and then screened using the pRevTRE-Lac Z vector for transfection and β-galactosidase assays to assess the tetracycline repressor function in different BT-474pRevTet-Off cell clones infected with pRevTet-Off (Fig.11).

The pRevTRE vector utilizes a hygromycin resistance gene, and selection of cells infected with pRevTRE-dn-3 on hygromycin allowed us to derive cell lines that expressed the tetracycline repressor protein as well as the dominant negative HER-3 gene driven by the CMV-TRE fusion promoter. PA-317 packaging cells were transfected with pRevTRE or pRevTRE-dn3, selected on hygomycin and these cell lines were then used to infect the tetracycline repressor-positive BT-474pRevTet-Off C3 cell clone with pRevTRE or pRevTRE-dn3. The infected cells were all cultured continuously in the presence of 10 ug/ml tetracycline to keep the dominant negative HER-3 gene off during their selection on hygromycin, cloning and passaging in culture prior to the execution of experiments. We isolated 78 different BT-474pRevTRE-dn3 cell clones, 38 of

A




B

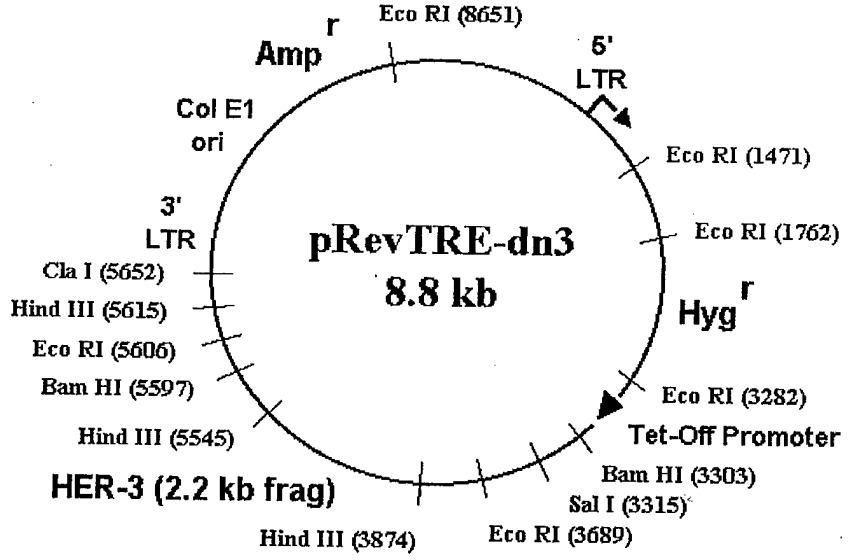



Fig. 9. *Tetracycline-repressible vectors for the expression of β-Galactosidase or dominant negative HER-3.* The pRevTRE tetracycline-repressible retroviral expression vector (Clonetech) was used to construct pRevTRE-Lac Z (A) and pRevTRE-dn3 (B). pRevTRE-Lac Z was used to transfect cell clones previously infected with pRevTet-Off (which contains the tetracycline repressor gene) and screen for tetracycline repressor activity. pRevTRE-dn3 was then introduced into tetracycline repressor-positive cell clones which were then cultured in the presence of tetracycline to keep the ectopic gene off during selection on hygromycin.

| | | Predicted Fragment Length (kb) | |
|---|---|---|---|
| **Lane** | **Enzyme** | **pRevTRE-Lac Z** | **pRevTRE-dn3** |
| 1) | Bam HI | 6.6, 3.8 | 6.5, 2.3 |
| 2) | Cla I | 9.6, 0.8 | 8.8 |
| 3) | Eco RI | 3.6, 3.3, 1.6, 1.5... | 3.1, 1.9, 1.6, 1.5... |
| 4) | Hind III | 10.4 | 7.1, 1.7... |
| 5) | Sal I | 6.6, 3.8 | 8.8 |

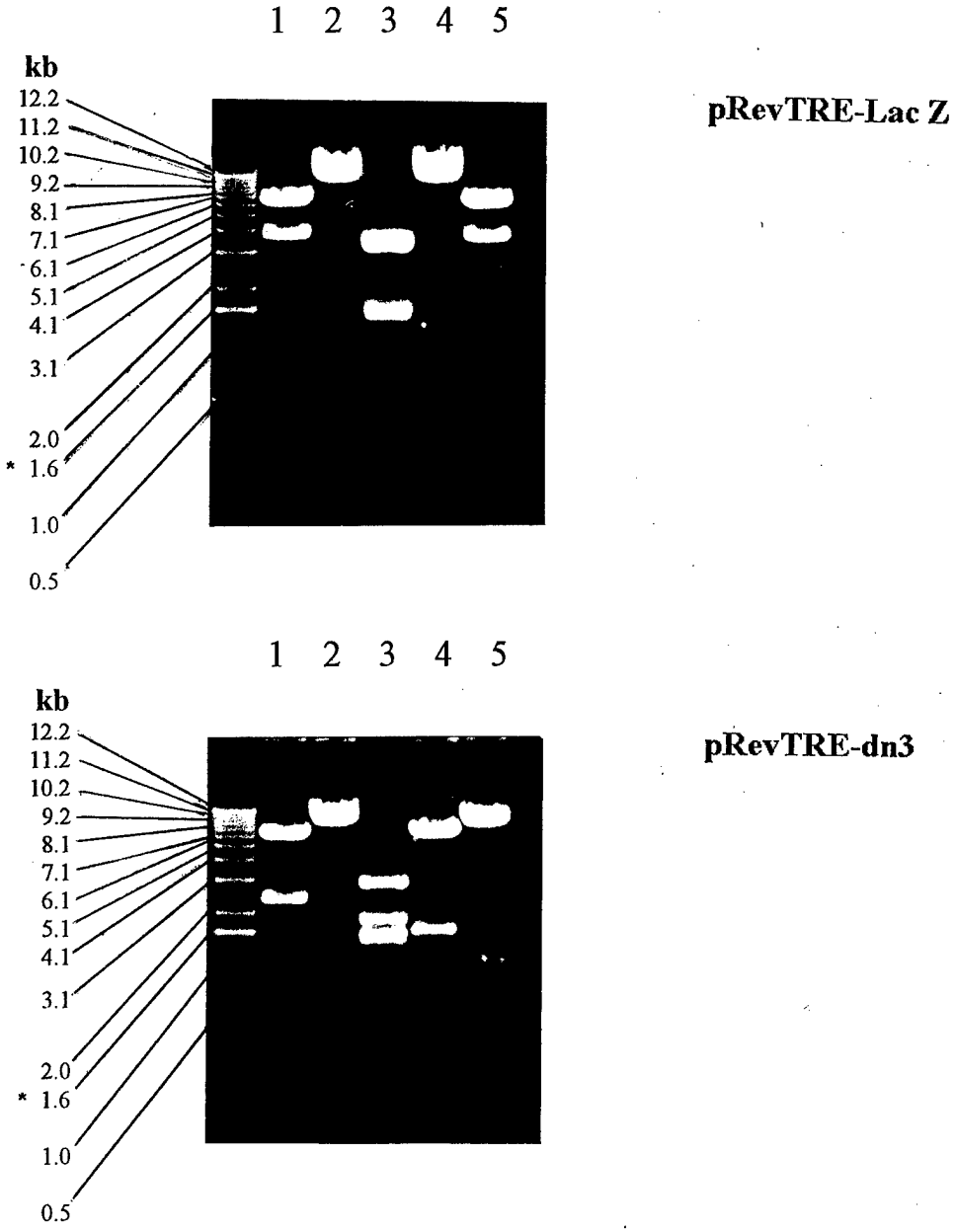



Fig. 10. *Restriction digest analysis of pRevTRE-LacZ and pRevTRE-dn3 retroviral expression vectors.* Restriction digests of plasmid DNA were electrophoresed in 0.8% agarose gels containing 0.5% ethidium bromide and photographed using UV light. A 1 kb DNA ladder is shown and the 1.6 kb marker (*) is the brightest band in the ladder.

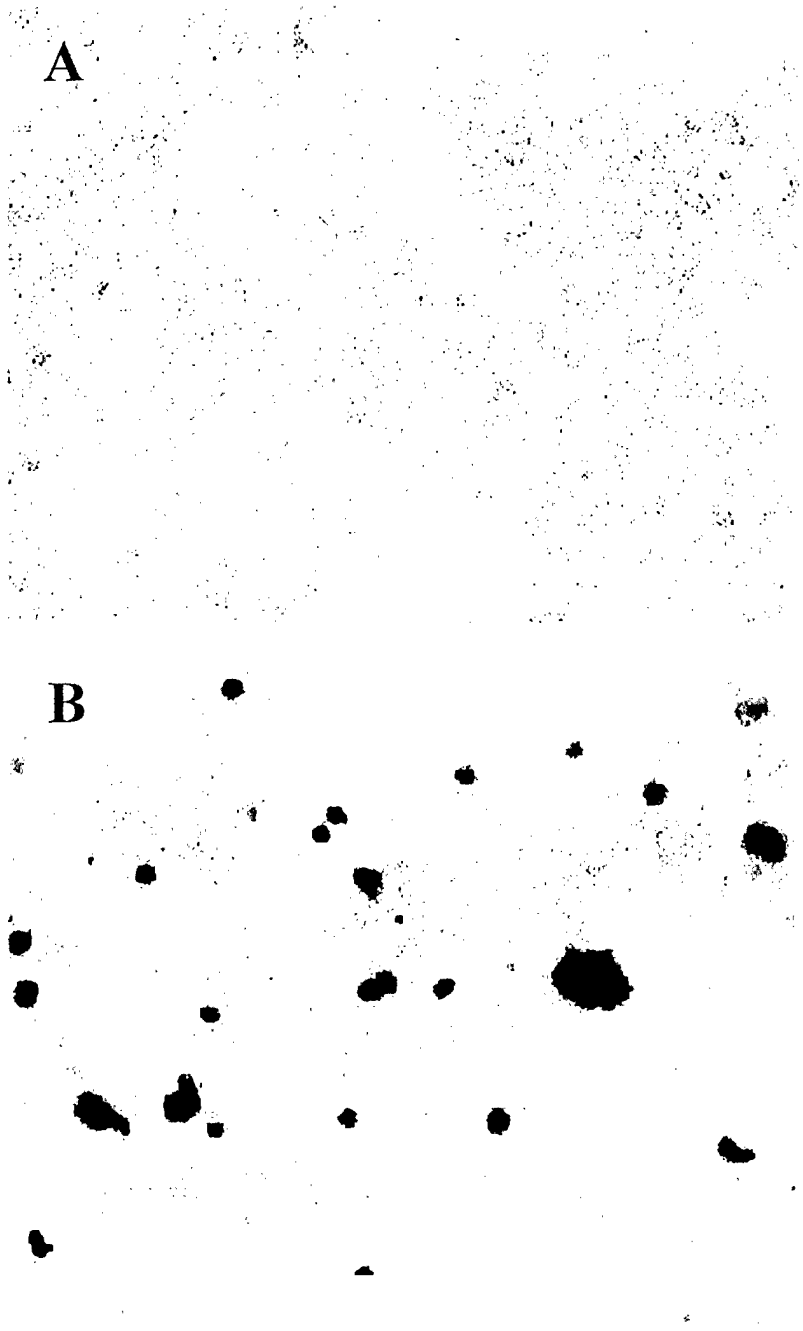



Fig. 11. *β-galactosidase activity in BT-474pRevTet-Off cells with or without tetracycline.* In order to screen for tetracycline repressor activity in different BT-474pRevTet-Off cell clones, BT-474pRevTet-Off cells were transiently transfected with pRevTRE-Lac Z and cultured either with (A) or without (B) tetracycline prior to staining for β-galactosidase activity. Bright field microscopy of BT-474pRevTet-Off C3 cells stained for β-Galactosidase activity showed tetracycline repressor function when the marker gene was not expressed in the presence of tetracycline. This cell clone was then used for further infection with pRevTRE-dn3 in order to generate BT-474 cells that express a regulatable dominant negative HER-3 gene.

which were successfully passaged for further use in the presence of tetracycline during the third year of the grant period. These experiments involved extensive time selecting, passaging and screening of a large number of clones and thus took much of the third year of the grant period to complete. BT-474pRevTet-Off C3 cells infected with pRevTRE and selected on hygromycin were used as a control. Prior to the start of experiments (at least 10 days), tetracycline was removed from one set of the cells, while the others were continuously cultured with tetracycline. We then screened a number of the BT-474pRevTRE-dn3 cell clones for the expression of dominant negative HER-3 as well as the level of tyrosine-phosphorylated HER-2/HER-3. Immunocytochemistry was used to screen cell clones for the expression of HER-3 both in the presence and absence of tetracycline. Western blot analysis was also used to measure the levels of dominant negative HER-3 and tyrosine-phosphorylated HER-2/HER-3 in the different cell clones. We succefully isolated a number of BT-474pRevTRE-dn3 cell clones that showed the induction of dominant negative HER-3. For example, the BT-474pRevTRE-dn3 C49 cell clone showed a high level of dominant negative HER-3 induction as well as a concomitant reduction in tyrosine-phosphorylated HER-2/HER-3 in the absence of tetracycline (Fig. 12).

*Growth of BT-474 Cells with Inducible Dominant Negative HER-3 in Culture and In Vivo*

Growth experiments were performed both in culture and *in vivo* using the BT-474pRevTRE-dn3 C49 cell clone and were carried out during the end of the third year of the grant period and completed during the period of the grant extension. Unlike 21MT-1 cells, BT-474 cells cannot be cultured under serum-free conditions. However, monolayer growth experiments were carried out using conditions including 10% FBS and different growth factors and BT-474pRevTER-dn3 cells showed significant reduction in growth compared to control BT-474pRevTRE cells in such experiments (data not shown). BT-474 cells have been reported to grow in soft agarose (51), so experiments were also conducted using these cell lines in soft agarose to assess their level of anchorage-independent growth in culture (Fig. 13). The growth of BT-474pRevTRE-dn3 cells was significantly reduced in soft agarose for cells cultured in the absence of tetracycline ($P < 0.05$) (Fig. 13). This showed that the regulatable expression of dominant negative HER-3 in BT-474 cells inhibited the transformed growth of these cells in culture.

*In vivo* experiments were also conducted using the BT-474pRevTRE and BT-474-pRevTRE-dn3 cells during the end of the third year of the grant period and were completed during the grant extension. Twenty-four injections were performed for each cell line using the same procedures as described above. In this case, 1 mouse injected with BT-474pRevTRE cells died during injection, and thus 20 injections for the control cells grew out for 2 full months before autopsy was performed. Additionally, 2 of the mice injected with BT-474pRevTRE-dn3 cells died from unrelated causes about halfway through the 2 month period. These mice showed no tumors at the time of autopsy, but were not included in the data set because they had not survived the full 2 months. The resulting data presented for injections from the mice that survived the full 2 months clearly showed a highly significant reduction of tumor growth for BT-474pRevTRE-dn3 cells compared to the control BT-474pRevTRE cells ($P < 0.05$) (Fig. 14). The average tumor volumes were 44.6 +/- 34.5 $mm^3$ for BT-474pRevTRE cells ($n = 20$) compared to 1.9 +/- 3.6 $mm^3$ for the pRevTRE-dn3 cells ($n=16$) [or for visible tumors, 7.7 +/- 2.2 $mm^3$ for BT-

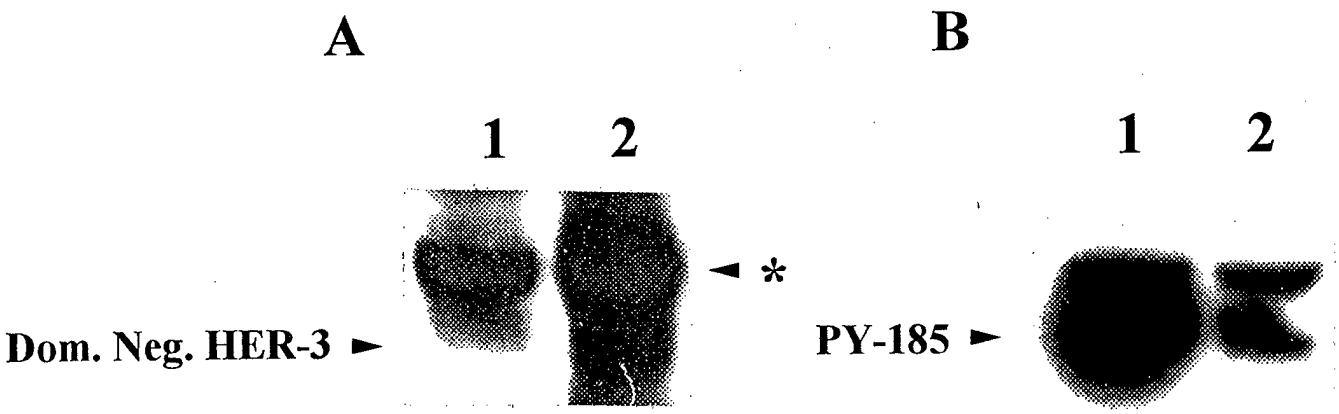



Fig. 12. *Expression of dominant negative HER-3 and tyrosine phosphorylation of HER-2/HER-3 in BT-474pRevTRE-dn3 C49 cells.* A) Samples containing 100 ug cell lysate protein per lane were immunoblotted with the polyclonal anti-HER-3 antibody for the BT-474pRevTRE-dn3 C49 cells with (Lane 1) and without (Lane 2) 10 ug/ml tetracycline. The tetracycline was removed 2 weeks prior to protein extraction and the serum was also removed 48 hours prior to protein extraction. The polyclonal anti-HER-3 antibody raised to the extracellular region of HER-3 was used to identify the approximately 100 kD dominant negative HER-3 protein which is seen in the same region as a non-specific band that is marked ( * ) on the right. B) Duplicate samples were also immunoblotted with the anti-phosphotyrosine monoclonal antibody for the BT-474pRevTRE-dn3 C49 cells with (Lane 1) and without (Lane 2) 10 ug/ml tetracycline to measure the relative levels of tyrosine-phosphorylated HER-2/HER-3.

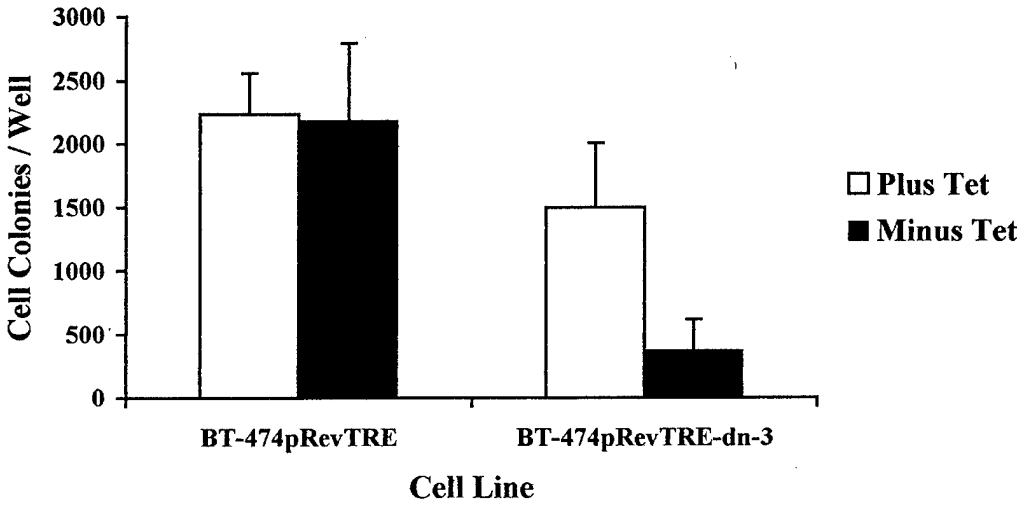
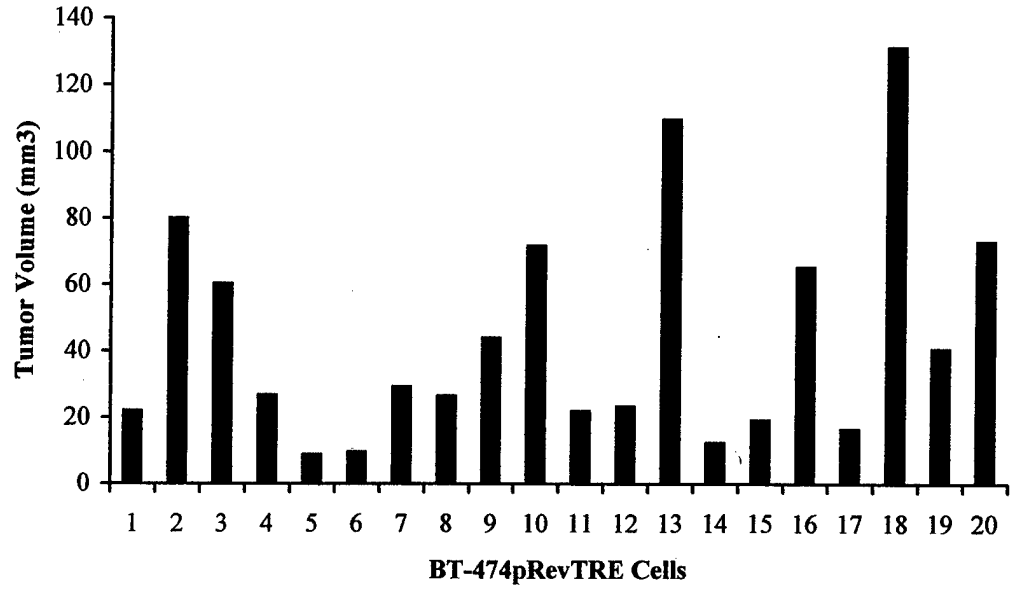



Fig. 13 *Anchorage-independent growth of BT-474pRevTRE and BT-474pRevTRE-dn3 C49 cells in soft agarose culture.* Cells were plated in 0.3% agarose and cultured with DMEM + 10% FBS plus insulin and EGF for 1 month before counting colonies at least 100 uM in diameter. The mean average and standard deviation for triplicate samples is shown. BT-474pRevTRE-dn3 C49 cells showed a significant reduction in colony number in the absence of tetracycline compared to cells with tetracycline ($p < 0.05$), or compared to control cells in the absence of tetracycline ($p \leq 0.05$). The StatMost program (Dataxiom Software Inc) was used to calculate significance in paired t-Tests.



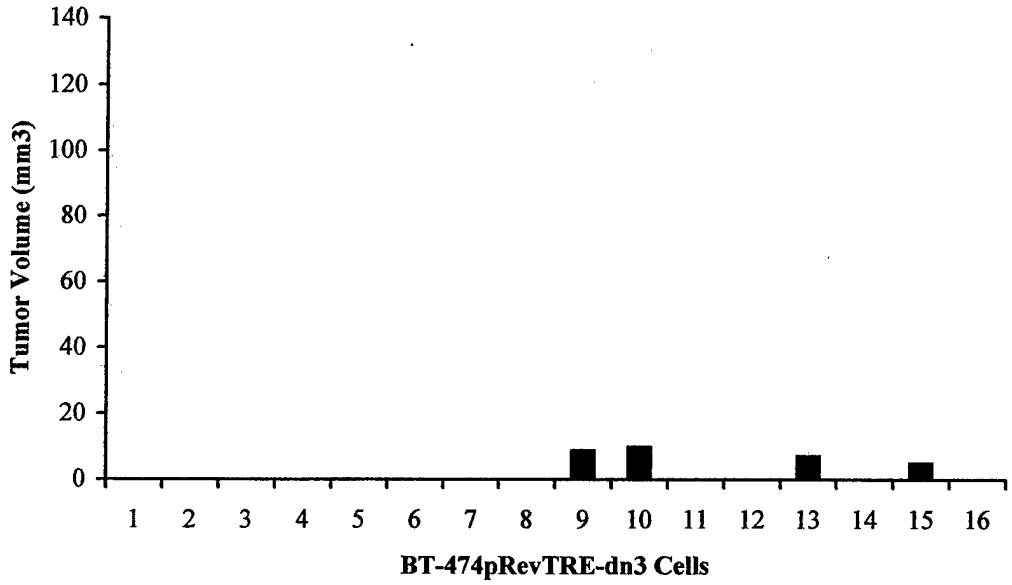




Fig. 14. Tumor growth of BT-474pRevTRE-dn3 C49 cells in SCID mice. Histogram showing the distribution of tumors for BT-474pRevTRE and BT-474pRevTRE-dn3 C49 cells grown for 2 months in female SCID mice. Cells ($10^7$) were injected s.c. in a solution containing 50% Matrigel and the tumors were removed after they had grown for 2 months *in vivo*, measured with vernier calipers, and tumor volumes (in $mm^3$) were calculated using the formula [volume = 0.52 x $(width)^2$ x (length)] according to Bergers et al., 2000 (50). The results showed a highly significant reduction in tumor growth for BT-474pRevTRE-dn3 cells compared to BT-474pRevTRE cells ($P < 0.05$) grown in SCID mice. The StatMost program (Dataxiom Software Inc) was used to calculate significance in paired t-Tests.

474pRevTRE-dn3 cells (n= 4)]. As mentioned above, because of the small size of BT-474 tumors, and especially for the BT-474pRevTRE-dn3 cell tumors, more extensive biochemical characterization of tumors as outlined in the original grant proposal was not possible employing this model system.

*Summary*

All of the objectives and specific tasks outlined in the original grant proposal have been completed. Brief descriptions including explicit reference to the specific tasks in the original statement of work as well as the periods of time in which the work was actually performed and completed are summarized below.

Task 1: (completed)

The construction and initial characterization of all of the cell lines that were infected with pCMV or pCMV-dn3 and mass selected was performed as outlined in original grant proposal and was completed by the end of the first year of the grant period. In addition, a series of BT-474pCMV-dn3 cell clones were also isolated that expressed the dominant negative HER-3 during the second year of the grant period. BT-474 cell lines were also constructed that express inducible dominant negative HER-3, and extensive passaging and screening of a large number of clones was performed to complete this work throughout the second and third year of the grant period.

Task 2: (completed)

The initial characterization of the expression of dominant negative HER-3 in the various mass selected cell populations infected with pCMV or pCMV-dn3 was completed by the end of the first year of the grant period. Similar characterization was performed for the various BT-474-derived cell clones as well as the inducible clones infected with pRevTRE or pRevTRE-dn3 during the second and third year of the grant period.

Task 3: (completed)

The measurement of receptor levels, activation and signaling was fully examined using the 21MT-1 cells as well as in BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV or pCMV-dn3, as outlined in the original grant proposal. This work was completed during the first half of the grant period. Measurement of receptor levels and activation was also performed for the various BT-474-derived cell clones as well as for the inducible clones during the second and third year of the grant period.

Task 4: (completed)

Much of this work was completed early on using the 21MT-1-derived cell lines for extensive serum-free tissue culture studies. The determination of the growth properties of BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV or pCMV-dn3 was completed by the end of the second year of the grant period. Extensive characterization of the culture growth properties of BT-474 cells infected with pRevTRE or pRevTRE-dn3 was carried out during the third year of the grant period and was completed during the grant extension.

Task 5: (completed)

The initial determination of the tumorigenicity for the various cells lines used in these studies were completed during the first two years of the grant period. Because the BT-474 cells showed the most reliable tumorigenicity, these cells were then used for the subsequent experiments *in vivo*. Tumor studies using BT-474 cells infected with pCMV and pCMV-dn3 were completed by the second year of the grant period. Tumor studies utilyzing the BT-474pRevTRE and BT-474pRevTRE-dn3 cells was carried out during the third year of the grant period and completed during the grant extension.

Task 6: (completed)

Basic histological examination and immunocytochemistry was performed on BT-474 cell tumors where it was possible during the third year of the grant and during the grant extension. However, due to the small size of the tumors attained, (and in particular for the BT-474pRevTRE-dn3 cells) more extensive biochemical characterization of tumors as outlined in the original grant proposal was not possible given the constraints of the model system that we employed.

## KEY RESEARCH ACCOMPLISHMENTS

- The 21MT-1-derived cell lines were used for extensive studies of the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture and *in vivo*.

- BT-474, MDA-MB-453 and SK-BR-3 cells were infected with pCMV and pCMV-dn3 and a number of BT-474-derived cell clones infected with pCMV-dn3 were also isolated for in-depth characterization of the effects of dominant negative HER-3 on these cell lines.

- BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV and pCMV-dn3 were characterized for the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture and *in vivo*.

- Additional vectors and cell lines were developed to study the effects of regulatable dominant negative HER-3 gene expression in BT-474 cells using the "Tet-Off" system.

- BT-474 infected with pRevTRE and pRevTRE-dn3 cells were characterized for their expression of dominant negative HER-3, the activation of HER-2/HER-3 and their growth responses in culture and *in vivo*.

## REPORTABLE OUTCOMES

- A manuscript describing most of the work done with the H16N-2 and 21MT-1 cell lines was published after submission and approval of the grant proposal (see attachment).

- A poster describing work performed throughout the first 2 years of the grant period was presented at the 2002 Department of Defense "Era of Hope" meeting (see attachment).

- A manuscript is presently in preparation containing additional data using the 21MT-1 cells as well as the BT-474, MDA-MB-453 and SK-BR-3 cells infected with pCMV or pCMV-dn3, and will also include recent data attained using the BT-474 cells infected with pRevTRE or pRevTRE-dn3 using the "Tet-Off" system.

## CONCLUSIONS

Amplification and overexpression of the HER-2 gene is seen in 28% of primary breast carcinomas. In those individuals with HER-2 gene amplification, this dominant genetic event is likely to be the principle element that drives malignancy because HER-2 is such a potent oncogene when highly overexpressed in experimental systems. However, our understanding is still fragmentary concerning the exact mechanisms by which signals for cell growth are constitutively activated in breast cancer cells with HER-2 gene amplification, and methods for permanently inhibiting the constitutive activation of signaling in such cells have not yet met with great success. Our previous work showed that the high-level overexpression of HER-2 in 21MT-1 breast cancer cells was associated with the constitutive activation of HER-2/HER-3, PI 3-kinase, and growth factor independence in culture (34, 45). By constitutively activating key mitogenic signaling pathways to a level that is effective for autonomous growth, tumor cells are thought to escape the normal controls on cell cycle regulation. Therefore, we sought to experimentally assess the importance of the cooperative interactions between HER-2 and HER-3 during the growth factor-independent proliferation of breast cancer cells with HER-2 gene amplification. Our data now confirm the importance of the HER-2/HER-3 heterodimer interaction for the constitutive receptor activation and recruitment of key signaling molecules in 21MT-1 cells. Dominant negative HER-3 also potently inhibited the growth factor-independent and anchorage-independent growth of 21MT-1 cells in culture (46). Furthermore, the expression of an inducible form of dominant negative HER-3 in BT-474 cells was able to inhibit anchorage-independent growth in soft agarose culture as well as tumorigenesis in SCID mice *in vivo* (manuscript in preparation). Therefore, our work studying the interaction between HER-2 and HER-3 offers exciting new opportunities for blocking the mechanisms of autonomous growth in breast cancer cells with HER-2 gene amplification.

By itself, HER-3 is known to be almost completely kinase deficient (as would be expected from sequence analysis showing deleterious substitutions in the enzymatic site) and is not able to activate signaling in-and-of-itself in genetically engineered cell lines that do not co-express any of the other HER kinases. However, while the other HERs have active kinase domains, HER-3 contains multiple additional docking sites for PI 3-kinase and SHC proteins not found in the other HERs. HER-2 is also known to be an especially active tyrosine kinase that exhibits ligand-independent activation when overexpressed. These combined considerations (i.e. HER-3 docking sites combined with HER-2 kinase potential) may account for the especially potent activation of signaling induced by HER-2/HER-3 heterodimers in response to HRG in H16N-2 cells (34, 47) and that is constitutively activated in 21MT-1 cells (34). Interestingly, the blocking of HER-2/HER-3 function with dominant negative HER-3 showed specificity in that H16N-2 and 21MT-1 cells infected with pCMV-dn3 and selected on G418 still proliferated in response to exogenous EGF. This suggested that HER-1/HER-3 is not as effectively inhibited by dominant negative HER-3 as is HER-2/HER-3 at a given level of dominant negative HER-3 gene expression, or that the interaction between EGFR and HER-3 is not required for EGF-induced proliferation. As mentioned above, HER-1 and HER-3 interact to some extent in these (47) and other cell lines (30-31), and this interaction may be required for EGF-stimulated growth. However, the relative affinity of HER-1/HER-3 heterodimers is very weak compared to HER-2/HER-3 heterodimers when compared with cross-linking analysis (42-44). Therefore, in order to further investigate the preferential effects that dominant negative HER-3 had on HER-2/HER-3-mediated growth, we

also compared the effects of dominant negative HER-3 on both HRG- and EGF-induced activation of HERs with anti-phosphotyrosine immunoblotting. While dominant negative HER-3 inhibited the levels of tyrosine-phosphorylated HER-2/HER-3 in 21MT-1 cells, the EGF-induced tyrosine phosphorylation of EGFR-containing dimers was not apparently affected by dominant negative HER-3. This result was also consistent with the hypothesis that EGFR-containing dimers are not as effectively inhibited by dominant negative HER-3 as are HER-2/HER-3 heterodimers at a given level of dominant negative HER-3 in genetically engineered cell lines.

We originally proposed using BT-474, MDA-MB-453 and SK-BR-3 cells as well as 21MT-1 cells for our studies because they are all breast carcinoma cell lines that have been reported to have amplification of HER-2 as well as constitutive activation of HER-2/HER-3 (32-34). While the 21MT-1 cells were useful for the characterization of the effects of dominant negative HER-3 in cell culture studies, the inability of these cells to form tumors presents a serious limitation in their use for more extensive studies *in vivo*. It is not entirely clear yet why many of the available breast carcinoma cell lines are not tumorigenic in immunodeficient mice, but apparently this is a common problem that has been previously reported to some extent (48). Interestingly, many previous reports studying HER-2-mediated cell transformation employed fibroblasts genetically engineered to overexpress HER-2 rather than breast carcinoma cell lines with HER-2 amplification for their *in vivo* studies. So far, we have found the BT-474 cells to be the most reliably tumorigenic in SCID mice, so we have focused most of our attention on this cell line for the tumorigenesis studies *in vivo*. We found that BT-474 cells also express the highest levels of HER-2 and the highest levels of constitutively activated HER-2/HER-3 and signaling. While most of the available human breast tumor cell lines, including 21MT-1, MDA-MB-453 and SK-BR-3 cells, have been derived from pleural effusions, BT-474 cells were uniquely derived from solid invasive ductal carcinoma (51). It is not yet known if this may be relevant, but it is striking to note that the other cell lines were all isolated from pleural effusions and had apparently adapted to conditions essentially growing in suspension, while BT-474 cells were growing as a solid tumor prior to isolation.

We generated BT-474, MDA-MB-453 and SK-BR-3 cells stably infected with either pCMV or pCMV-dn3, and screened the cells for the expression of dominant negative HER-3. We used these cells and other newly developed BT-474 cell clones infected with pCMV-dn3 to further study the effects of dominant negative HER-3 on HER-2/HER-3 activation, signaling and growth responses in culture and *in vivo*. However, while we were able to generate BT-474, MDA-MB-453 and SK-BR-3 cells infected with these vectors, we also routinely saw a much lower number of cell colonies growing out during their initial selection on G418 for the cells infected with pCMV-dn3 compared to those infected with pCMV under otherwise identical conditions in culture. Furthermore, we noticed that many of the colonies that started to grow out in plates infected with pCMV-dn3 did not continue to grow, suggesting that the proliferation and survival of many of the cell clones infected with pCMV-dn3 was inhibited by the expression of dominant negative HER-3. This is also consistent with the extensive heterogeneity in anti-HER-3 staining seen in mass selected and extensively passaged cell populations. In addition, we have now found that cells infected with pCMV-dn3 and stained with immunocytochemistry immediately after being selected on G418 showed high-level expression of dominant negative HER-3 in many of the slower growing and/or dying colonies. The low number of colonies seen for cells infected with pCMV-dn3 indicated that the growth of many of the cells expressing the highest levels of dominant negative HER-3 may be inhibited during their initial selection on

antibiotic. Therefore, most cells infected with pCMV-dn3 appeared to form small or attenuated colonies, while other clones expressing lower levels of dominant negative HER-3 (but still apparently enough to be antibiotic-resistant) may be selected for during subsequent passaging in culture. This would clearly alter the stoichiometric relationship between these receptor proteins on the cell surface, possibly generating cell populations with lower mutant/wild-type ratios and less inhibition of wild-type function.

Little is known about the exact growth factor requirements for BT-474, MDA-MB-453, and SK-BR-3 cells, which are cultured under undefined conditions in medium containing 10% fetal calf serum. Therefore, it may also be difficult to maintain cell populations that express high levels of dominant negative HER-3 under conditions where constitutive and regulative growth responses have not yet been determined. Due to stochiometric considerations, it is crucial to generate cells that express very high levels of the dominant negative HER-3 in order to effectively block wild-type HER-2/HER-3 function. As mentioned above, if the highest level expressing cells are being selected out because of the inhibitory effects of dominant negative HER-3, this may necessitate the use of a regulatable expression system to study the full extent of the effects of dominant negative HER-3 in these cells. The tetracycline-repressible (i.e. " Tet-Off") pRevTRE retroviral expression vector facilitates the inhibition of ectopic genes in mammalian cells during the selection on hygromycin in the presence of tetracycline (Clontech). Therefore, we constructed a tetracycline-repressible dominant negative HER-3 retroviral expression vector. The newly constructed vector was then used to infect BT-474 cells that had previously been infected with pRevTet-Off, selected on G418 and screened for functional tetracycline repressor activity by transfection of the pRev-TRE-Lac Z vector and culture with or without tetracycline prior to assessing the level of β-galactosidase activity. The pRevTRE vector utilizes a hygromycin resistance gene, and by infecting Tet-Off-infected cell lines with pRevTRE-dn3 and selecting them on hygromycin as well as G418, this then allowed us to derive cell clones that express the tetracycline transcriptional regulator protein as well as the dominant negative HER-3. Cells infected with the regulatable form of dominant negative HER-3 were kept in the presence of tetracycline to keep the dominant negative HER-3 gene off during selection on hygromycin. Cells with and without tetracycline were then used for experiments to measure the levels of dominant HER-3 and its effectiveness in inhibiting HER-2/HER-3 activation and growth responses in culture. Extensive tumor studies were then also carried out to compare the growth of BT-474pRevTRE and BT-474pRevTRE-dn3 cells in SCID mice *in vivo*. This "Tet-Off" system then allowed us to maintain the cells with the ectopic gene off in the presence of tetracycline and, with removal of tetracycline, turn the dominant negative HER-3 gene on just prior to the start of the experiments. It was anticipated that this may help reduce the potential effects of selection pressure against dominant HER-3 gene expression in infected cell lines during the initial selection on antibiotic and during their routine passage in culture. Recent data attained using BT-474pRevTRE-dn3 cells that express the inducible dominant negative HER-3 has now shown dramatically that, under these conditions, dominant negative HER-3 was able to inhibit HER-2/HER-3 activation as well as the transformed growth of BT-474 cells both in culture and *in vivo*.

The feasibility of success for strategies involving gene therapy are greatly improved by our ability to introduce genes into cells using retroviral-mediated infection of cells. Constructing the dominant negative HER-3 vector was an especially important focus for us, because this had not been previously attempted and now appears to be effective in preferentially blocking the heterodimer interactions between HER-2 and HER-3 provided the levels of the ectopic gene are

sufficiently high in target cell lines. This strategy of blocking HER-2/HER-3 with a dominant negative form of HER-3, combined with retroviral-mediated gene transfer technology, offers potential for further studying HER-2/HER-3 activation in breast cancer cells. However, much of our data also indicated that stoichiometric considerations must be carefully evaluated as well. As mentioned above, previous studies employing dominant negative vectors for other receptor tyrosine kinases, such as PDGF, FGF and EGF receptors, have met with great success in blocking the ligand-induced activation of those receptors for cells in culture (38-40). Some of their studies also indicated that the stoichiometric aspect of the mutant/wild-ratio was very important to achieve effective blocking in their studies. Interestingly, the complexities of how selective pressure may allow cancer cells to overcome growth inhibition through adaptation and progressive selection of cells with lower levels of ectopic gene expression may relate to mechanisms by which cancer cells could also potentially subvert the inhibitory effects of genes during gene therapy.

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## APPENDICES

- Ram, T. G., Schelling, M., and Hosick, H. L. Blocking HER-2/HER-3 function wth a dominant negative form of HER-3 in cells stimulated by heregulin and in cancer cells with HER-2 gene amplification. Cell Growth & Diff. 11:173-83, 2000.

# Blocking HER-2/HER-3 Function with a Dominant Negative Form of HER-3 in Cells Stimulated by Heregulin and in Breast Cancer Cells with HER-2 Gene Amplification[1]

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## Abstract

**Amplification and overexpression of the HER-2 (*neu*/*erb*B-2) gene in human breast cancer are clearly important events that lead to the transformation of mammary epithelial cells in approximately one-third of breast cancer patients. Heterodimer interactions between HER-2 and HER-3 (*erb*B-3) are activated by *neu* differentiation factor/heregulin (HRG), and HER-2/HER-3 heterodimers are constitutively activated in breast cancer cells with HER-2 gene amplification. This indicates that inhibition of HER-2/HER-3 heterodimer function may be an especially effective and unique strategy for blocking the HER-2-mediated transformation of breast cancer cells. Therefore, we constructed a bicistronic retroviral expression vector (pCMV-dn3) containing a dominant negative form of HER-3 in which most of the cytoplasmic domain was removed for introduction into cells. By using a bicistronic retroviral vector in which the antibiotic resistance gene and the gene of interest are driven by a single promoter, we attained 100% coordinate coexpression of antibiotic resistance with the gene of interest in target cell populations. Breast carcinoma cells with HER-2 gene amplification (21 MT-1 cells) and normal mammary epithelial cells without HER-2 gene amplification from the same patient (H16N-2 cells) were infected with pCMV-dn3 and assessed for HER-2/HER-3 receptor tyrosine phosphorylation, p85$^{PI\ 3\text{-}kinase}$ and SHC protein activation, growth factor-dependent and -independent proliferation, and transformed growth in culture. Dominant negative HER-3 inhibited the HRG-induced activation of HER-2/HER-3 and signaling in H16N-2 and 21 MT-1 cells as well as the constitutive activation of HER-2/HER-3 and signaling in 21 MT-1 cells. Responses to exogenous HRG were strongly inhibited by dominant negative HER-3. In contrast, the proliferation of cells stimulated by epidermal growth factor was not apparently affected by dominant negative HER-3. The growth factor-independent proliferation and transformed growth of 21 MT-1 cells were also strongly inhibited by dominant negative HER-3 in anchorage-dependent and -independent growth assays in culture. Furthermore, the HRG-induced or growth factor-independent proliferation of 21 MT-1 cells was inhibited by dominant negative HER-3, whereas the epidermal growth factor-induced proliferation of these cells was not: this indicates that dominant negative HER-3 preferentially inhibits proliferation induced by HER-2/HER-3.**

## Introduction

The HER-2 (*neu*/*erb*B-2) gene encodes a $M_r$ 185,000 protein tyrosine kinase that is highly homologous to the EGF[3] receptor (HER-1/EGFR/*erb*B-1), HER-3 (*erb*B-3), and HER-4 (*erb*B-4; Refs. 1–3), which together comprise the type 1 family of receptor tyrosine kinases (4, 5). The HER family receptor tyrosine kinases all contain ectodomains with two cysteine-rich sequences. Despite this structural homology, these receptors differ in their ligand specificities (4). Thus, HER-1 binds several ligands closely related to EGF, whereas HER-3 and HER-4 are the receptors for a number of different isoforms of *neu* differentiation factor/HRG (6–8). Whereas no direct ligand for HER-2 has yet been cloned, it is now clear that HER-2 is capable of heterodimerization with HER-1 (9, 10), HER-3 (11, 12), or HER-4 (8), and these HER-2-containing heterodimers form the highest affinity binding sites for their respective ligands (10, 11). HER-2 is amplified in 28% of primary breast carcinomas *in vivo* (13), and another 10% of primary breast carcinomas overexpress HER-2 without amplification of the gene (14–16). In addition, HER-2 gene amplification concordant with high-level overexpression is associated with increased tumor aggressiveness and the poor prognosis of breast cancer patients (13, 14, 17–19). Other related genes, such as the HER-1 gene, are sometimes amplified in human breast cancers (13). However, amplification of the HER-1 gene is much less common than that seen for HER-2 (2% *versus* 28%, respectively) in breast cancer. Whereas amplification of HER-3 or HER-4 has not been seen in various studies (2, 3), our own work and the studies of

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[3] The abbreviations used are: EGF, epidermal growth factor; BSA, bovine serum albumin; HRG, *neu* differentiation factor/heregulin; IGF, insulin-like growth factor; PI 3-kinase, phosphatidylinositol 3-kinase; SF, serum-free; TBS, Tris-buffered saline; TTBS, Tween TBS; HRP, horseradish peroxidase; DAB, diaminobenzidine; TTBS, 150 mM NaCl, 50 mM Tris (pH 7.5), and 0.1% Tween 20.

others (20–23) have now shown that heterodimer interactions between HER-2 and HER-3 are constitutively activated in breast cancer cells with HER-2 gene amplification, and cotransfection of HER-3 with HER-2 greatly augments the transforming capability of HER-2 in genetically engineered cell lines (21). HER-2/HER-3 heterodimer complexes are now thought to potently activate the PI 3-kinase and mitogen-activated protein kinase signal transduction pathways to a level that is effective for transformation. We are particularly interested in how the cooperative effects of HER-2 and HER-3 activate various mitogenic signal transduction pathways involved in cell growth.

Experimentally elevated HER-2 gene expression in various cell lines, including nontransformed human mammary epithelial cells, induces the complete transformation of cells injected into nude mice (24–27). The potent oncogenic potential of HER-2 is generally thought to be due to its ability to constitutively activate various key signal transduction pathways that are involved in the regulation of cell growth. However, whereas our current understanding of the oncogenic potential of HER-2 has expanded quite rapidly (for review, see Ref. 28), our knowledge of exactly how HER-2 induces the neoplastic transformation of human mammary epithelial cells still remains fragmentary. For example, although HER-2 was originally discovered as the *neu* transmembrane-mutated form of the gene in rat neuroblastoma cells (29), the HER-2 gene found in human breast cancer has never shown such mutations (30), but the level of tyrosine-phosphorylated HER-2 in primary human breast cancer *in vivo* always shows a direct correspondence with the overexpression of HER-2 (31). This suggests that high-level overexpression of wild-type HER-2 alone is sufficient to constitutively activate its tyrosine kinase function. Furthermore, the protein encoded by the wild-type HER-2 gene was also previously shown to possess constitutive tyrosine kinase activity if sufficiently overexpressed in a variety of cell lines in culture in the absence of any identifiable ligand (24–27, 32, 33), and transfection of a gene encoding a chimeric receptor containing the HER-1 extracellular domain fused to the cytoplasmic domain of HER-2 results in the constitutive tyrosine kinase activity of the chimeric receptor in the absence of EGF (32, 33). This indicates that the tyrosine kinase domain of HER-2 exhibits a greater tendency toward ligand-independent activation than do the other HERs when overexpressed.

Another area of great importance concerns the heterodimeric associations that are now known to occur between the different HER proteins, including HER-1 and HER-2 (9, 10), HER-2 and HER-3 (11, 12), HER-2 and HER-4 (8), and HER-1 and HER-3 (34, 35) in response to ligands. Our own work and that of others (20–22) has now established that the heterodimer interactions between HER-2 and HER-3 are also constitutively activated in breast cancer cells with HER-2 gene amplification, and the cooperative interactions between HER-2 and HER-3 are associated with the constitutive activation of various signaling pathways in cancer cells with HER-2 gene amplification. However, the involvement of HER-2/HER-3 heterodimers in the constitutive activation of signaling pathways that transform cancer cells with HER-2 gene amplification has not yet been tested with perturbative analysis. One strategy that has been used successfully to block the function of other receptor tyrosine kinases uses dominant negative expression vectors in which the region coding for the cytoplasmic domain of the receptor is almost completely removed. Although the truncated receptor still contains the transmembrane domain and can thus dimerize within the cell, it lacks tyrosine kinase activity and inhibits the signal transduction docking function. This strategy has been used effectively to block HER-1 (36), platelet-derived growth factor receptor (37), and fibroblast growth factor receptor (38). Recently, a dominant negative HER-2 vector was also used successfully to block HER-2 function in normal mouse development (39). The use of such HER-2 vectors has apparently not yet been useful for blocking HER-2 function in cancer cells with HER-2 gene amplification, probably due to the stoichiometric problems associated with the inability to generate mutant/wild-type levels high enough for effective inhibition. However, the fact that HER-3 is not highly overexpressed in these cells and that activated HER-2 and HER-3 have a particularly high affinity interaction (40–42) suggests that dominant negative HER-3 may be especially effective in blocking HER-2/HER-3 function.

## Results

**H16N-2 and 21 MT-1 Cells Provide a Model System for Studying the Role of HER-2/HER-3 in HRG-induced Mitogenesis and the Autonomous Growth of Cancer Cells with HER-2 Gene Amplification.** For these studies, we used cell lines originally isolated from a patient with infiltrating and intraductal carcinoma of the breast with HER-2 gene amplification (43, 44). The 21 MT-1 metastatic breast carcinoma cell line was isolated from a pleural effusion collected during an advanced stage of the disease. H16N-2 cells are nonneoplastic cells isolated from normal mammary tissue of the same patient; thus, they serve as an ideal control for studying the 21 MT-1 cells as well as the effects of HRG in nontransformed cells. RFLP analysis had previously shown that these cell lines share common genetic polymorphisms (43), and we have also verified that the H16N-2 and 21 MT-1 cells are derived from a single individual by DNA fingerprinting analysis of a hypervariable region of the BRCA-1 locus.[4] We have previously shown that the amplification and high-level overexpression of HER-2 in the 21 MT-1 cells is associated with HER-2/HER-3-mediated activation of PI 3-kinase and growth factor independence (*i.e.*, autonomous growth) in SF culture (22, 45). This system is ideal for studying receptor activation and signaling under well-defined conditions that allow us to distinguish constitutive from externally mediated growth factor responses in culture. To directly measure the activation of HER-2 and HER-3 in these cells, we starved the cells of growth factors for 48 h in SF medium and then directly extracted the cells (Fig. 1, *A* and *B*, *Lanes 1* and *3*) or stimulated the cells with HRG-$\beta$ for 10 min before extraction (Fig. 1, *A* and *B*, *Lanes 2* and *4*). Immunoprecipitation followed by immunoblotting directly showed the levels of

[4] Unpublished observations.

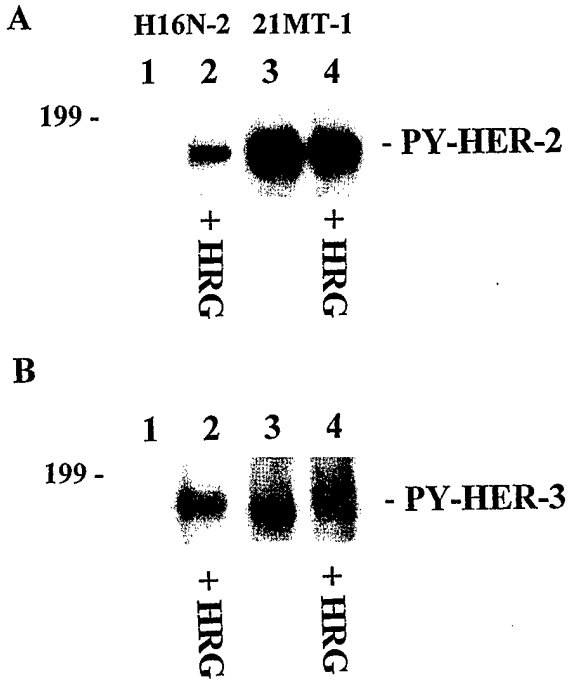


*Fig. 1.* Activation of HER-2/HER-3 by HRG and its constitutive activation in breast cancer cells with HER-2 gene amplification. Samples containing 2 mg of cell lysate protein were immunoprecipitated with anti-phosphotyrosine antibody, followed by immunoblotting with anti-HER-2 antibody (*A*) or immunoprecipitation with anti-HER-3 antibody followed by immunoblotting with anti-phosphotyrosine antibody (*B*) to show the level of HER-2 and HER-3 activated in cells with or without HRG stimulation in culture. H16N-2 nontransformed human breast epithelial cells (*Lanes 1* and *2*) and 21 MT-1 metastatic breast carcinoma cells with HER-2 gene amplification (*Lanes 3* and *4*) were starved of growth factors for 48 h in SF medium and then directly extracted (*Lanes 1* and *3*) or stimulated with 10 ng/ml HRG-$\beta$ for 10 min at 37°C before extraction (*Lanes 2* and *4*).

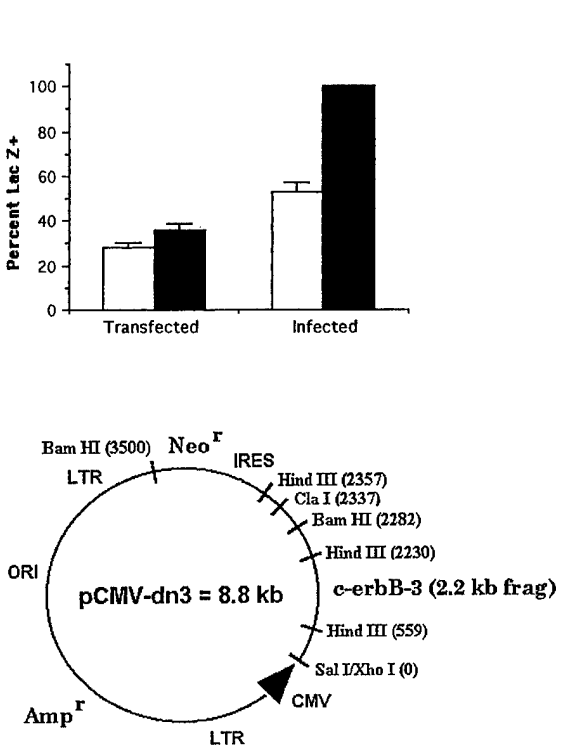


*Fig. 2.* Development of the dominant negative HER-3 bicistronic retroviral expression vector. *A*, to test for the efficiency of antibiotic resistance gene and marker gene coexpression in our cell lines, both monocistronic (double promoter) and bicistronic (single promoter) forms of a retroviral expression vector containing the Neo$^r$ and LacZ$^+$ genes were either transfected into or used to infect H16N-2 cells. Colonies selected on G418 for a month were then stained for $\beta$-galactosidase activity and counted to determine the percentage of blue-stained colonies. The mean average ± SD for triplicate wells is shown. □, monocistronic; ■, bicistronic. *B*, the pCMV-dn3 bicistronic retroviral expression vector was constructed containing a dominant negative form of the HER-3 gene in which most of the cytoplasmic domain of HER-3 was removed. This vector also contains an internal ribosome-binding site (*IRES*) between the HER-3 gene and the Neo$^r$ gene located downstream, which together form a single transcription unit. The expression of the pCMV-dn3 bicistronic transcript in mammalian cells then allows for the efficient coordinate coexpression of the dominant negative HER-3 gene with antibiotic resistance in retroviral-infected cell populations.

HER-2 and HER-3 activated in these cells. HRG induced tyrosine phosphorylation of both HER-2 and HER-3 in H16N-2 cells (Fig. 1, *A* and *B, Lane 2*), whereas 21 MT-1 cells show high-level constitutive activation of both HER-2 and HER-3 in the absence of exogenous growth factors in culture (Fig. 1, *A* and *B, Lane 3*) due to the amplification and overexpression of HER-2 in these cells (22, 45).

**Construction of H16N-2 and 21 MT-1 Cell Lines Expressing Dominant Negative HER-3.** We previously found that the introduction of standard monocistronic expression vectors (in which the antibiotic resistance gene and marker gene are driven by separate promoters) into many different cell lines did not lead to very efficient coordinate coexpression of antibiotic resistance with the gene of interest. Experiments were performed using retroviral control vectors containing the Neo$^r$ and LacZ$^+$ genes placed in either monocistronic or bicistronic configuration and then either transfected or infected into target cell lines to assess the LacZ$^+$ gene expression in G418-selected cell colonies (Fig. 2*A*). The results showed that cells infected with the bicistronic retroviral vector (in which the LacZ$^+$ and Neo$^r$ genes form a single transcription unit driven by one promoter) coordinately coexpressed antibiotic resistance with LacZ$^+$ expression in 100% of the G418-selected cell colonies. Thus, the infection of bicistronic retroviral vectors completely eliminated the occurrence of false positive clones in genetically engineered cells and resulted in greater efficiency of LacZ$^+$ gene expression within cell clones as well (data not shown). These results attained using the H16N-2 cells are also similar to those seen for a number of different mammary epithelial cell lines, including the 21 MT-1 cells (data not shown). Therefore, we used the pCMV bicistronic retroviral vector to express dominant negative HER-3 in target cells. By using the pBK-CMV phagemid expression vector (Stratagene) as an intermediate, we cloned a dominant negative HER-3 fragment into the pCMV bicistronic retroviral expression vector using flanking restriction sites located within the extensive polylinker region of pBK-CMV. The human HER-3 cDNA was used to clone a 2.2-kb fragment of HER-3 lacking most of the cytoplasmic domain into pBK-CMV to generate pBK-CMV-dn3, and this ligation also introduced an in-frame stop codon

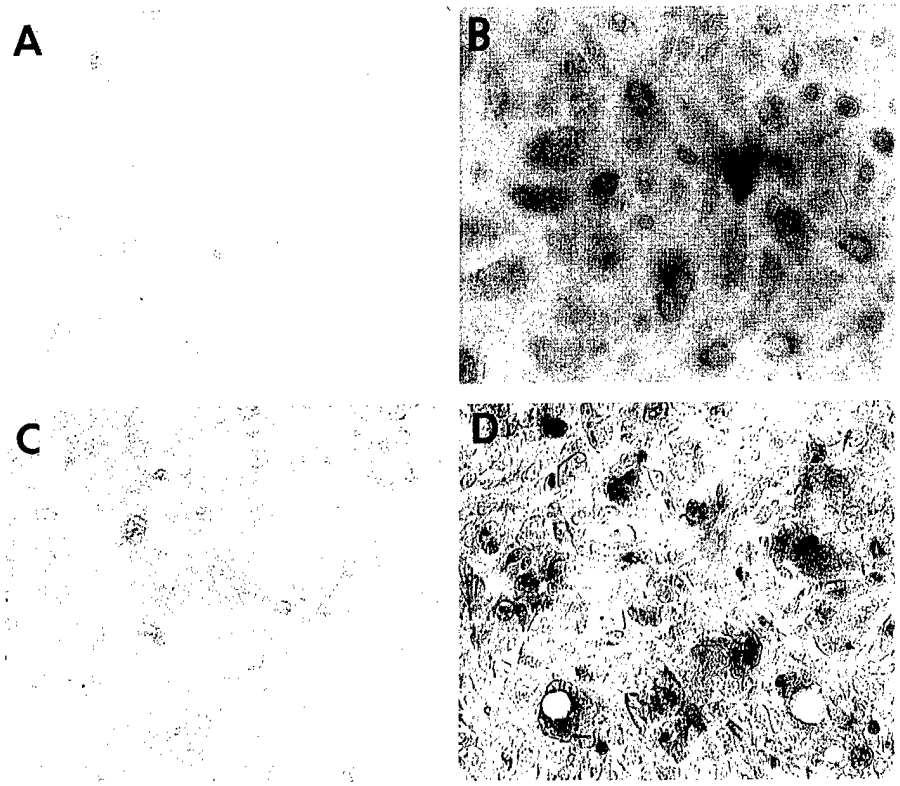


*Fig. 3.* Expression of dominant negative HER-3 in cells infected with pCMV-dn3. Immunocytochemistry with anti-HER-3 monoclonal antibody was used to measure the level of HER-3 protein in H16N-2 cells (*A*), H16N-2-dn3 cells (*B*), 21 MT-1 cells (*C*), and 21 MT-1-dn3 cells (*D*). The H105 antibody is known to be highly specific for HER-3 and binds to an epitope within the extracellular domain. Although control cells express wild-type HER-3 (Fig. 6), the levels are below the level for immunodetection with HRP/DAB staining. Therefore, the easily detectable levels of HER-3 measured in H16N-2-dn3 and 21 MT-1-dn3 cells confirmed the ectopic expression of dominant negative HER-3 in cells infected with pCMV-dn3.

12 codons downstream of the point of ligation. The dominant negative HER-3 insert removed from pBK-CMV-dn3 was then cloned into pCMV to generate pCMV-dn3 (Fig. 2*B*). Restriction digest analysis confirmed the proper construction of the vectors (data not shown). H16N-2 and 21 MT-1 cells were then infected with the pCMV backbone (used as a control) or pCMV-dn3 using the ψCRIP packaging cell line. The cell lines infected with the control vector are referred to as H16N-2 and 21 MT-1 cells, whereas those infected with pCMV-dn3 are referred to as H16N-2-dn3 and 21 MT-1-dn3 cells. Immunocytochemistry was performed to confirm that the H16N-2-dn3 and 21 MT-1-dn3 cells express the dominant negative HER-3 (Fig. 3). Expression of the ectopic HER-3 protein was assessed using the H105 anti-HER-3 monoclonal antibody that binds specifically to an epitope within the extracellular domain of HER-3. Although H16N-2 and 21 MT-1 cells express wild-type HER-3, the wild-type HER-3 protein levels are below the level for immunodetection with HRP/DAB staining using immunocytochemistry (Fig. 3, *A* and *C*). Therefore, the easily detectable levels of HER-3 measured in H16N-2-dn3 and 21 MT-1-dn3 cells (Fig. 3, *B* and *D*) readily confirmed the expression of dominant negative HER-3 in cells infected with pCMV-dn3.

**Inhibition of HER-2/HER-3 Activation in Cells Expressing Dominant Negative HER-3.** We next measured the effects of dominant negative HER-3 on the activation of HER-2/HER-3 in anti-phosphotyrosine immunoblots (Fig. 4*A*). Dominant negative HER-3 potently inhibited the HRG-induced tyrosine phosphorylation of HER-2/HER-3 in H16N-2-dn3 and 21 MT-1-dn3 cells (Fig. 4*A*, *Lanes 4* and *8*) as well as the constitutive tyrosine phosphorylation of HER-2/HER-3 in the 21 MT-1-dn3 cells (Fig. 4*A*, *Lane 7*). We also separately measured the levels of tyrosine-phosphorylated HER-2 and HER-3 by immunoprecipitation followed by immunoblotting (Fig. 5), which showed that dominant negative HER-3 inhibited HER-2 recruitment in anti-phosphotyrosine immunoprecipitates (Fig. 5*A*) and almost completely blocked HER-3 tyrosine phosphorylation (Fig. 5*B*) in H16N-2-dn3 and 21 MT-1-dn3 cells. Furthermore, immunoblots probed for HER-2 or HER-3 showed no significant effect of dominant negative HER-3 on the level of the wild-type HER-2 or HER-3 in H16N-2-dn3 and 21 MT-1-dn3 cells (Fig. 6), indicating that the effects of dominant negative HER-3 in H16N-2-dn3 and 21 MT-1-dn3 cells do not involve other effects on the expression of wild-type HER-2 or HER-3.

**Inhibition of HER-2/HER-3-mediated Signaling in Cells Expressing Dominant Negative HER-3.** We next measured the effects of dominant negative HER-3 on the recruitment and tyrosine phosphorylation of the signaling molecules, $p85^{PI\ 3\text{-}kinase}$, $p46^{SHC}$, and $p52^{SHC}$ in cells with and without HRG stimulation in culture. The dominant negative HER-3 was found to inhibit the recruitment of $p85^{PI\ 3\text{-}kinase}$ in anti-phosphotyrosine immunoprecipitates (Fig. 7). We have previously shown that this assay is a very reliable measure of the recruitment and activation of $p85^{PI\ 3\text{-}kinase}$ by HER-2/HER-3 (22). We (22) and others (46–48) have also found previously that activation of PI 3-kinase by various receptor tyrosine kinases involves recruitment of PI 3-kinase but does not involve detectable tyrosine phosphorylation of $p85^{PI\ 3\text{-}kinase}$ under more physiological conditions where $p85^{PI\ 3\text{-}kinase}$ is not artificially overexpressed (46).

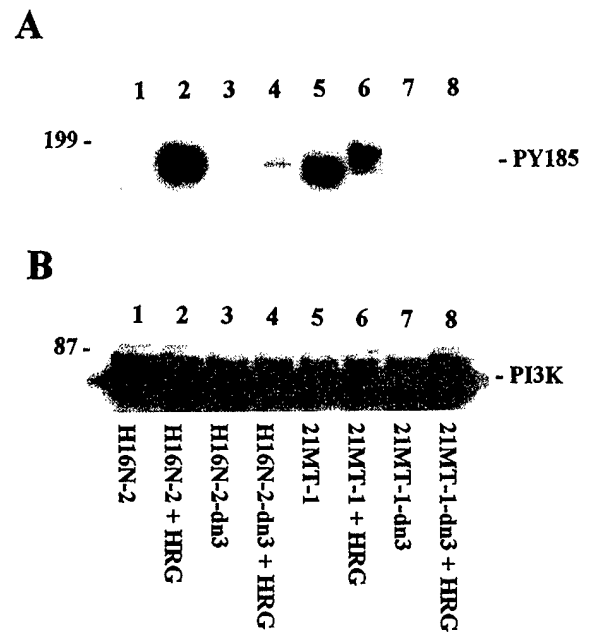


*Fig. 4.* Inhibition of HER-2 and HER-3 activation in cells expressing dominant negative HER-3. *A*, samples containing 100 μg of cell lysate protein were immunoblotted with anti-phosphotyrosine antibody. *B*, the same blot was then reprobed with anti-p85 antibody as a control to confirm equal loading of the gel. H16N-2 cells (*Lanes 1* and *2*), H16N-2-dn3 cells (*Lanes 3* and *4*), 21 MT-1 cells (*Lanes 5* and *6*), and 21 MT-1-dn3 cells (*Lanes 7* and *8*) were starved of growth factors for 48 h in serum-free medium and then directly extracted (*Lanes 1, 3, 5,* and *7*) or stimulated with HRG-β before extraction (*Lanes 2, 4, 6,* and *8*).

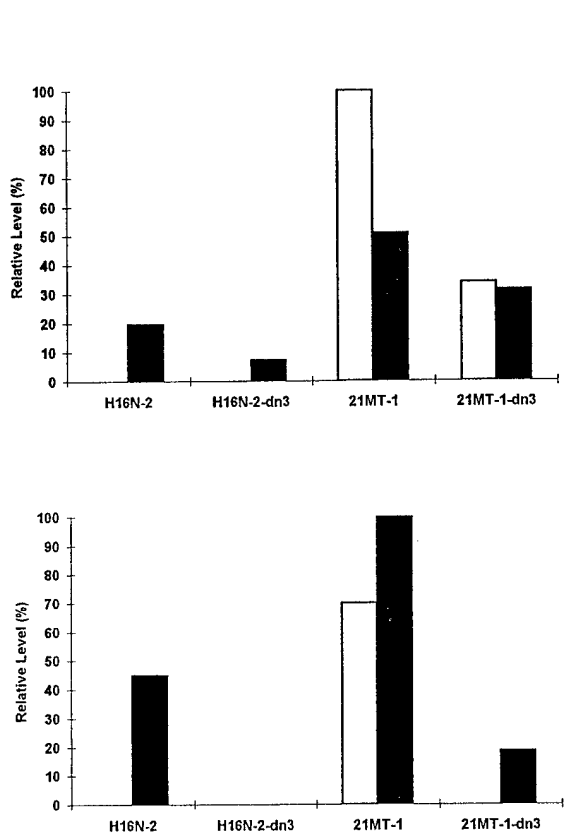


*Fig. 5.* Dominant negative HER-3 inhibition of HER-2 and HER-3 tyrosine phosphorylation. Immunoprecipitation followed by immunoblotting was used to separately determine the levels of tyrosine-phosphorylated HER-2 (*A*) and HER-3 (*B*) in the different cell lines with or without stimulation with HRG-β performed exactly as that shown in Fig. 1. The results shown here were attained from scanning densitometry of negatives exposed by chemiluminescent substrate. □, SF; ■, SF + HRG.

Therefore, the changes measured in the recruitment of $p85^{PI\ 3\text{-}kinase}$ in anti-phosphotyrosine immunoprecipitates reflect the level of PI 3-kinase recruited by activated receptor complexes (22). Furthermore, dominant negative HER-3 inhibited the recruitment of $p46^{SHC}$ and $p52^{SHC}$ in anti-phosphotyrosine immunoprecipitates (Fig. 8). However, in the case of SHC proteins, which are known to be highly tyrosine-phosphorylated during activation, the level in anti-phosphotyrosine immunoprecipitates likely reflects the combined effects on the tyrosine phosphorylation of SHC proteins as well as the level recruited by activated receptor complexes. In summary, the cells expressing dominant negative HER-3 showed impaired HER-2/HER-3 function as well as significant reductions in the recruitment and tyrosine phosphorylation of signaling molecules for both the PI 3-kinase and mitogen-activated protein kinase signal transduction pathways.

**Dominant Negative HER-3 Inhibits HRG-induced Proliferation and the Autonomous Growth of Breast Cancer Cells with HER-2 Gene Amplification.** We routinely use the H16N-2 and 21 MT-1 cell lines for our studies because they were derived from the same patient and can be grown under completely defined SF conditions in culture. This well-defined system allows us to study growth factor responses as well as growth factor-independent (*i.e.*, autonomous) proliferation in culture in a manner that is not possible for other cell lines derived in high serum-containing conditions. Anchorage-dependent monolayer growth assays with and without exogenous growth factors showed that dominant negative HER-3 inhibited the HRG-induced proliferation of both H16N-2-dn3 and 21 MT-1-dn3 cells in culture (Fig. 10*A*). In contrast, dominant negative HER-3 had no apparent effect on the insulin/EGF-induced proliferation of H16N-2-dn3 and 21 MT-1-dn3 cells in culture (Fig. 10*A*). Furthermore, the proliferation of the 21 MT-1-dn-3 cells was completely blocked in the absence of exogenous growth factors in culture (Figs. 9 and 10*A*). These results indicate that dominant negative HER-3 preferentially inhibits only proliferation induced by HRG or the growth factor-independent proliferation of cells that that overexpress HER-2. Finally, soft agarose growth assays were also performed to assess the potential effects of dominant negative HER-3 on the anchorage-independent growth of 21 MT-1-dn3 cells in culture. Dominant negative HER-3 strongly blocked the transformed growth of 21 MT-1-dn3 cells in soft agarose and inhibited growth even with maximal activation of HER-2/HER-3 in the presence of exogenous HRG (Fig. 10*B*).

## Discussion

Amplification and overexpression of the HER-2 gene in human breast cancer are clearly important events that lead to

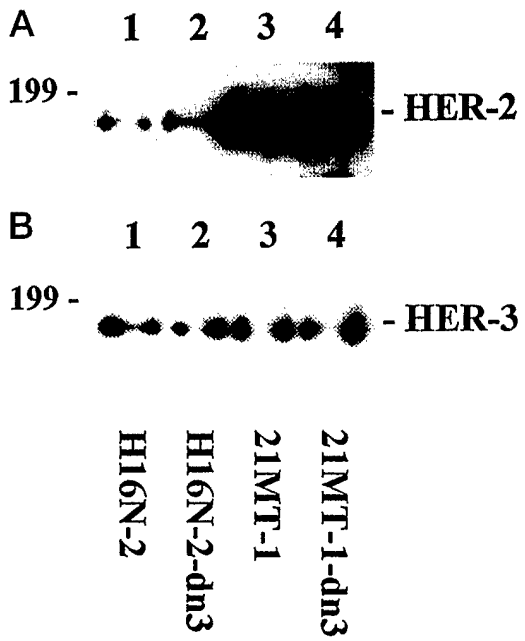


*Fig. 6.* Dominant negative HER-3 has no effect on the levels of endogenous HER-2 or HER-3. Samples containing 100 $\mu$g of cell lysate protein were immunoblotted with anti-HER-2 (*A*) or anti-HER-3 (*B*) antibody. H16N-2 cells (*Lane 1*), H16N-2-dn3 cells (*Lane 2*), 21 MT-1 cells (*Lane 3*), and 21 MT-1-dn3 cells (*Lane 4*) are shown.

the transformation of mammary epithelial cells in approximately one-third of breast cancer patients. In those individuals with HER-2 gene amplification, this dominant genetic event is likely the principle change that drives malignancy because HER-2 is such a potent oncogene when highly overexpressed in experimental systems. However, our understanding is still fragmentary concerning the exact mechanisms by which signals for cell growth are constitutively activated in breast cancer cells with HER-2 gene amplification, and methods for permanently inhibiting the constitutive activation of signaling in such cells have not yet met with great success. Our recent insights into the interaction between HER-2 and HER-3 offer exciting new opportunities for blocking the mechanism of autonomous growth in breast cancer cells with HER-2 gene amplification. By constructing cell lines that stably express a dominant negative form of HER-3, we have now successfully targeted the interaction between HER-2 and HER-3 in cells stimulated by exogenous HRG as well as that which is constitutively activated in breast cancer cells with HER-2 gene amplification. Also, the use of the pCMV-dn3 bicistronic retroviral vector results in highly efficient coordinate coexpression of antibiotic resistance and dominant negative HER-3 in target cell lines.

Our previous work showed that the elevated levels of HER-2 overexpression in 21 MT-1 cells are associated with high-level constitutive activation of PI 3-kinase and growth factor independence in culture (22, 45). In the present study, we sought to experimentally assess the importance of the cooperative interactions that occur between HER-2 and HER-3 in cells in response to HRG and during the growth factor-independent proliferation of breast cancer cells with HER-2 gene amplification. Our data now confirm the importance of the HER-2/HER-3 heterodimer interaction for recruiting key mitogenic signal transduction molecules involved in the growth of normal cells stimulated by HRG as well as in breast cancer cells with HER-2 gene amplification. Dominant negative HER-3 was able to block the HRG-induced proliferation of H16N-2 and 21 MT-1 cells as well as the growth factor-independent proliferation of 21 MT-1 cells in growth factor-free medium. In addition, dominant negative HER-3 potently inhibited the anchorage-independent growth of 21 MT-1 cells in soft agarose culture. These major effects of dominant negative HER-3 on cell proliferation do not necessarily preclude additional effects involving the rate of apoptosis in these cells, which remains to be determined. Also, preliminary *in vivo* studies have been performed using the 21 MT-1 and 21 MT-1-dn3 cells for injection into nude mice. However, to date, the 21 MT-1 control cell line has not been sufficiently tumorigenic in our nude mice to allow us to sufficiently test the effects of dominant negative HER-3 *in vivo*. The low tumor take and limited growth seen for 21 MT-1 cells in nude mice are apparently common problems for a significant number of highly malignant and metastatic breast carcinoma cell lines (49), and earlier studies with 21 MT-1 cells also suggested some difficulty in using these cells for tumor studies at later passages (50). Therefore, additional studies are under way using other breast carcinoma cell lines with HER-2 gene amplification as well as 21 MT-1 cells for transplantation into various immunodeficient mouse strains.

By itself, HER-3 is known to be almost completely kinase deficient (as would be expected from sequence analysis, which shows alterations in the enzymatic site) and is therefore unable to activate signaling in and of itself in genetically engineered cell lines that do not coexpress any of the other HER kinases (5, 40–42). However, whereas the other HERs have active kinase domains, HER-3 contains multiple additional docking sites for $p85^{PI\ 3\text{-}kinase}$ and SHC proteins not found in the other HERs (5). Also, as mentioned above, HER-2 is known to be an especially active tyrosine kinase that exhibits ligand-independent activation when overexpressed (33). These combined considerations (*i.e.*, HER-3 docking sites combined with HER-2 kinase potential) may account for the especially potent activation of signal transduction induced by HER-2/HER-3 heterodimers in response to HRG and seen constitutively in breast cancer cells (20–22). Interestingly, the blocking of HER-2/HER-3 function with dominant negative HER-3 was preferential in that the cells still proliferated in response to exogenous EGF, suggesting that the interaction between HER-1 and HER-3 is not necessary for mitogenesis in cells stimulated by EGF or that dominant negative HER-3 does not block HER-1/HER-3 function as well as HER-2/HER-3. In fact, it was this specificity of dominant negative HER-3 inhibition of HER-2/HER-3 that allowed us to use a constitutive promoter to express dominant negative HER-3, because the cells infected with pCMV-dn3 were still able to proliferate in response to EGF. Whereas there is evidence that HER-1 and HER-3 interact to some extent in these and other cell lines (34, 35, 51), the HER-1/HER-3 heterodimer interaction is clearly very weak compared to that for HER-2/HER-3 (40–42).

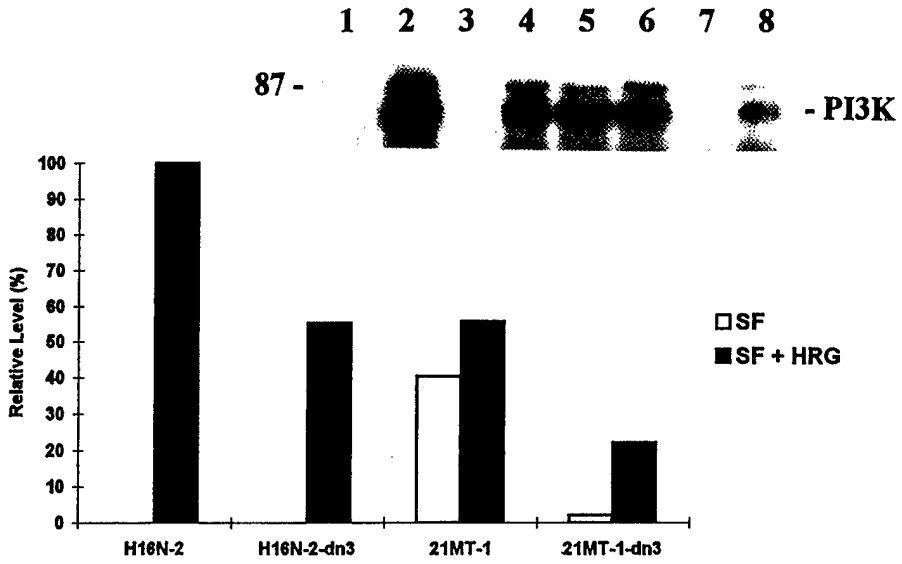


*Fig. 7.* Inhibition of PI 3-kinase recruitment by HER-2/HER-3 in cells expressing dominant negative HER-3. Samples containing 2 mg of cell lysate protein were immunoprecipitated with anti-phosphotyrosine antibody followed by immunoblotting with anti-p85 antiserum to show the level of $p85^{PI\ 3\text{-}kinase}$ recruited by tyrosine-phosphorylated receptor complexes. H16N-2 cells (*Lanes 1* and *2*), H16N-2-dn3 cells (*Lanes 3* and *4*), 21 MT-1 cells (*Lanes 5* and *6*), and 21 MT-1-dn3 cells (*Lanes 7* and *8*) were starved of growth factors for 48 h and then directly extracted (*Lanes 1, 3, 5,* and *7*) or stimulated with HRG-β before extraction (*Lanes 2, 4, 6,* and *8*).

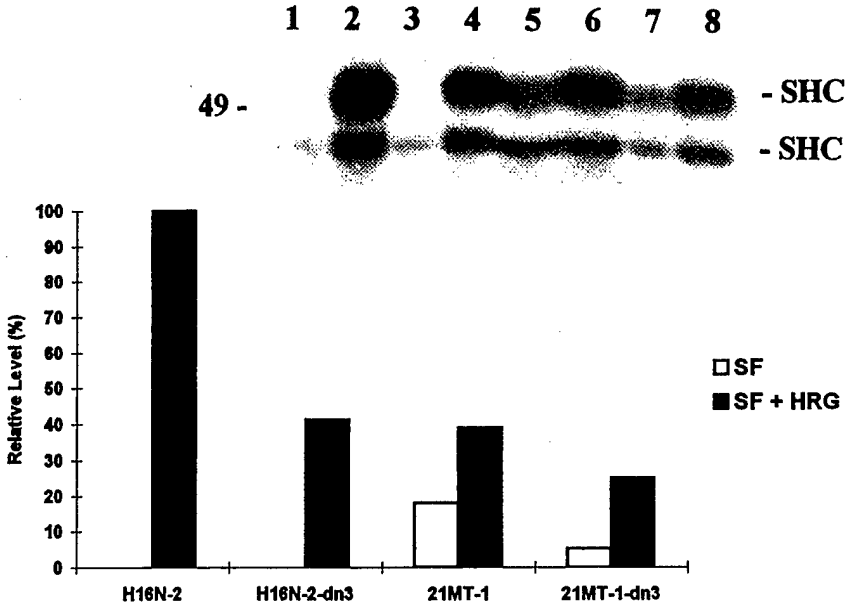


*Fig. 8.* Inhibition of SHC protein tyrosine phosphorylation and recruitment in cells expressing dominant negative HER-3. Samples containing 2 mg of cell lysate protein were immunoprecipitated with anti-phosphotyrosine antibody followed by immunoblotting with anti-SHC antiserum to show the level of $p46^{SHC}$ and $p52^{SHC}$ protein tyrosine phosphorylation and recruitment by tyrosine-phosphorylated receptor complexes. H16N-2 cells (*Lanes 1* and *2*), H16N-2-dn3 cells (*Lanes 3* and *4*), 21 MT-1 cells (*Lanes 5* and *6*), and 21 MT-1-dn3 cells (*Lanes 7* and *8*) were starved of growth factors for 48 h and then directly extracted (*Lanes 1, 3, 5,* and *7*) or stimulated with HRG-β before extraction (*Lanes 2, 4, 6,* and *8*).

Human breast carcinoma cells sometimes overexpress HER-3, and it has been suggested that this may be important for malignancy (2). However, whereas HER-3 is commonly expressed at a low but functional level in most nontransformed and transformed human mammary epithelial cells that we have tested, the HER-3 gene has never been found to be amplified or highly overexpressed, as is HER-2 (2). Furthermore, when cell lines are genetically engineered to overexpress HER-3, this alone is not sufficient to constitutively activate HER-3 or to transform cells (21). As mentioned above, HER-3 is a very weak kinase compared to the other HERs (5, 40–42), but HER-3 is constitutively activated in HER-2-overexpressing cell lines in which the cooperative interaction between HER-2 and HER-3 activates HER-2/HER-3 heterodimers (20–23). However, a number of the breast cancer cell lines with HER-2 gene amplification, such as 21 MT-1 cells, do not overexpress HER-3 in comparison with normal cells (Fig. 6). Therefore, it is our contention that low-level HER-3 cooperates with HER-2 to effectively transform breast carcinoma cells with HER-2 amplification, but this mechanism of cell transformation does not require concordant overexpression of HER-3.

Growth factor independence, as a phenotype, is a good indicator of progressive cell transformation in tumor cells with HER-2 gene amplification (22, 45). Normal human mammary epithelial cells require both IGF-I (or supraphysiological levels of insulin) and EGF to proliferate under

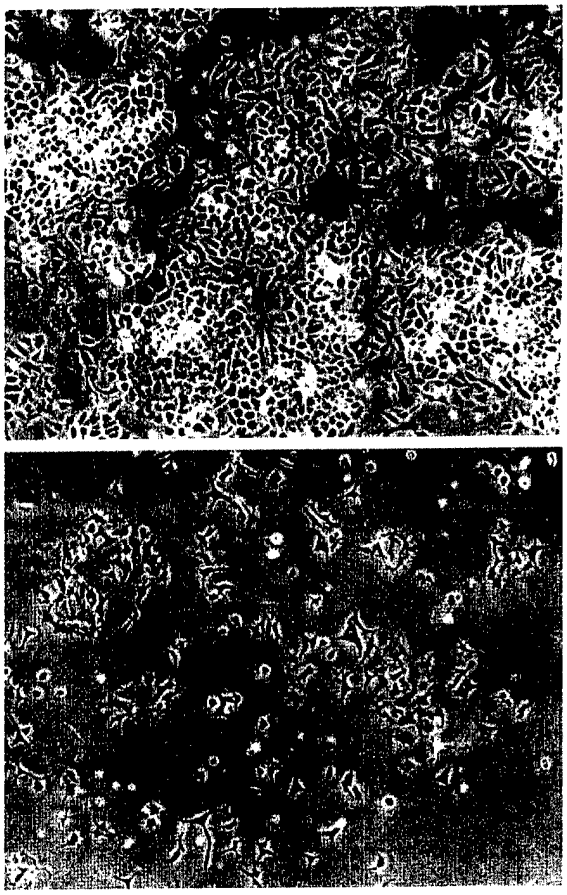

*Fig. 9.* Dominant negative HER-3 inhibits the autonomous proliferation of 21 MT-1 cells in monolayer culture. Phase-contrast microscopy of 21 MT-1 (*A*) and 21 MT-1-dn3 cells (*B*) cultured in SF medium in the complete absence of growth factors for 9 days is shown.

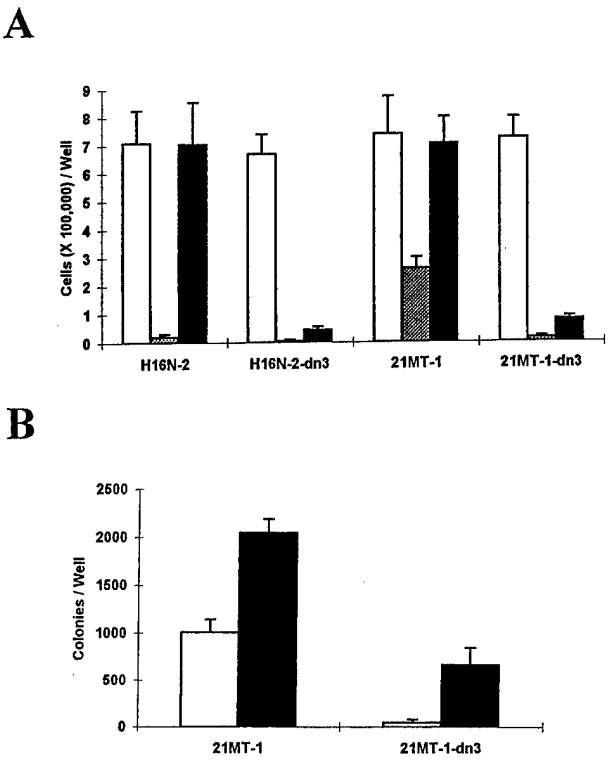


*Fig. 10.* Dominant negative HER-3 inhibits the HRG-induced proliferation of cells as well as the autonomous proliferation and transformed growth of 21 MT-1 cells in culture. *A*, anchorage-dependent growth assay showing the proliferation of H16N-2, H16N-2-dn3, 21 MT-1, and 21 MT-1-dn3 cells in monolayer culture for 9 days with SF medium plus insulin and EGF (□), without any growth factors (▨), or plus HRG-$\beta$ (■). The mean average ± SD for triplicate wells is shown. *B*, anchorage-independent growth assay of 21 MT-1 and 21 MT-1-dn3 cells cultured for a month in soft agarose with or without HRG-$\beta$. The mean average ± SD for triplicate wells is shown. □, medium; ■, medium + HRG.

SF conditions in culture (52). The synergistic requirement for both IGF and EGF in the proliferation of normal mammary epithelial cells suggests that the attainment of growth factor-independent proliferation in mammary carcinoma cells involves genetic changes that subvert requirements for both IGF and EGF. We have previously shown that the 21 MT-2 and 21 MT-1 breast carcinoma cell lines have equivalently amplified HER-2 but show progressively elevated levels of HER-2 transcription associated with increasing IGF and EGF independence in culture (23, 45). We also found that HRG substitutes for both IGF and EGF in stimulating the proliferation of nontransformed human mammary epithelial cells (which express both HER-2 and HER-3, but not HER-4) in culture (45, 52). Therefore, we previously proposed that HER-2/HER-3 constitutive activation of signaling pathways in breast cancer cells substitutes for growth factor-mediated signaling, which usually requires the combination of IGF and EGF in normal cells (22, 45, 52). Furthermore, the distinguishing properties of HER-2/HER-3 function may help explain the occurrence, and potent oncogenicity and selection of amplified HER-2 in cell types that normally express HER-3.

## Materials and Methods

**Vector Construction.** For experiments to test the efficiency of antibiotic resistance gene and marker gene coexpression in our cell lines, control retroviral expression vectors were constructed from the pCMV vector (originally derived from pSLH1001, which was derived from pLNCX) in which the Neo$^r$ and the LacZ$^+$ genes were placed in either monocistronic or bicistronic configuration. The dominant negative HER-3 retroviral expression vectors were made using full-length human HER-3 cDNA (Amgen, Inc.), from which a 2.2-kb fragment missing most of the intracellular domain was generated by cutting out the insert with *Sal*I and *Bam*HI. By using the pBK-CMV phagemid expression vector (Stratagene) as an intermediate, we subcloned the dominant negative HER-3 fragment into the pCMV bicistronic retroviral expression vector. We first ligated the dominant negative HER-3 fragment into the *Sal*I and *Bam*HI sites located within the extensive polylinker region of pBK-CMV to generate pBK-CMV-dn3. This ligation also introduced an in-frame stop codon 12 codons downstream of the *Bam*HI site. The dominant negative HER-3 fragment was then subcloned from pBK-CMV-dn3 into pCMV by ligation of the dominant negative HER-3 insert cut with *Sal*I and *Cla*I (which does not contain these sites internally) into the *Xho*I and *Cla*I sites (*Sal*I and *Xho*I have compatible ends) within pCMV to generate pCMV-dn3 (Fig. 2*B*). Restriction digest analysis confirmed the proper construction of the vectors.

**Culture Infection and Selection of Cell Lines.** The H16N-2 and 21 MT-1 cell lines were provided by Dr. Vimla Band through the Dana-Farber Cancer Institute (Boston, MA). For routine culture, the cells were grown in F-12 growth medium containing 10 mM *N*-2-hydroxyethylpiperazine-2-ethane sulfonic acid, antibiotic/antimycotic, 0.5 $\mu$g/ml fungizone, 5 mM

ethanolamine, 50 ng/ml sodium selenate, 1 μg/ml hydrocortisone, 5 μg/ml transferrin, 5 μg/ml insulin, 10 ng/ml EGF, 0.1 mg/ml BSA, and 2% fetal bovine serum. The cells were cultured at 37°C with 5% carbon dioxide, and the medium was changed every other day. For subculture, the cells were rinsed in calcium magnesium-free HBSS and then rinsed in 0.05% trypsin plus 0.025% EDTA in calcium magnesium-free HBSS. After aspiration of the trypsin solution, the cells were incubated at 37°C for 5–15 min, and the released cells were immediately resuspended in growth medium for replating in 60- or 100-mm tissue culture plates. For routine culture, the cells were counted with a hemocytometer and plated at a density of $10^4$ cells/cm$^2$. For experiments to test the efficiency of antibiotic resistance (Neo$^r$) and marker gene (LacZ$^+$) coexpression, monocistronic and bicistronic control vectors were introduced into H16N-2 and 21 MT-1 cells by either transfection or infection. For transfection, the DNA was introduced into cells by lipofection with Lipofectin according to the manufacturer's instructions (Life Technologies, Inc.). For infection, the vectors were first transfected into the ψCRIP packaging cell line followed by infection of target cells with replication-defective virus. Colonies selected for a month on 200 μg/ml G418 (Life Technologies, Inc.) were then fixed and stained for β-galactosidase activity, and the proportion of blue-stained colonies was determined from colony counts. H16N-2 and 21 MT-1 cells were then infected with either the pCMV vector (as a control) or the pCMV-dn3 vector. For these infections, ψCRIP cells were transiently transfected with either pCMV or pCMV-dn3 by lipofection, and medium conditioned for 24 h containing virus was collected and spun down at 1200 rpm for 10 min before adding to subconfluent H16N-2 and 21 MT-1 cell cultures. The H16N-2 and 21 MT-1 cells were then incubated with ψCRIP conditioned medium for 3 days, with fresh conditioned medium added daily. After an additional 2-day incubation in fresh medium, the infected cell lines were then selected on 200 μg/ml G418 for a month before use in further analysis.

**Immumocytochemistry.** The cells were plated in 24-well plates at a density of $5 \times 10^2$ cells/well and cultured to confluence. The cells were rinsed in PBS, fixed in methanol at −20°C for 10 min, and then rinsed three times with PBS before immunostaining with the anti-HER-3 monoclonal antibody, H105 (Neomarkers). The cells were equilibrated in TBS [150 mM NaCl and 50 mM Tris (pH 7.5)], blocked in TBS plus 1% BSA at room temperature for 60 min with mild agitation, and then with 2 μg/ml H105 antibody in TBS plus 1% BSA at room temperature for 60 min with mild agitation. The cells were then rinsed in TBS three times (5 min each time) with moderate agitation, incubated with biotinylated antimouse IgG secondary antibody (Vector Laboratories) at a 1:750 dilution in TBS plus 1% BSA at room temperature for 60 min with mild agitation, rinsed in TBS three times (5 min each time) with moderate agitation, and then incubated with ABC strepavidin HRP reagents (Vector Laboratories) diluted in TBS + 1% BSA at room temperature for 60 min with mild agitation. After rinsing of the cells in TBS three times (5 min each time) with moderate agitation, the cells were stained with DAB as the substrate.

**Immunoprecipitations.** Cells cultures were incubated in SF medium without insulin and EGF for 48 h before extraction (*i.e.*, the constitutive condition) and stimulation with 10 ng/ml HRG-β for 10 min at 37°C before extraction of lysate protein for immunoprecipitation and/or Western blot analysis. After the cells were lysed in immunoprecipitation buffer [150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% NP40, 5 mM EDTA, 5 mM sodium orthovanadate, 10 mM Na $PP_i$, and 2 mM phenylmethylsulfonyl fluoride], the lysates were clarified by centrifugation at 14,000 × g for 15 min and either used directly for electrophoresis or used for immunoprecipitation after normalizing the samples. Total cell lysate protein was assayed using the Bradford assay (Bio-Rad), and 2 mg of protein were used for immunoprecipitation, or 100 μg of protein were used directly for electrophoresis. For immunoprecipitation, cell lysates were then incubated with either 30 μl anti-phosphotyrosine monoclonal antibody-conjugated agarose (Oncogene) for 2 h at room temperature with moderate agitation or with 2 μg of 2F12 anti-HER-3 monoclonal antibody (Neomarkers) for 2 h at room temperature with moderate agitation followed by incubation with 50 μl of protein A-agarose (Oncogene) for 1 h at 4°C with moderate agitation. The pellets were then washed three times in immunoprecipitation buffer, and the beads were boiled in 100 μl of electrophoresis sample buffer for 10 min to release protein conjugates from the agarose before electrophoresis.

**Western Blot Analysis.** Cell lysates or immunoprecipitated samples were electrophoresed in 7.5% SDS-PAGE gels for approximately 18 h at 15 mA constant current. The samples were then transferred to Immobilon-P membranes (Millipore) by overnight electrotransfer in standard transfer buffer at 125 mA followed by 2 h at 325 mA. The blots were equilibrated in TTBS, blocked in TTBS plus 3% milk at room temperature for 60 min with moderate agitation, and then incubated with either 2 μg/ml PY20 anti-phosphotyrosine monoclonal antibody (Oncogene), 1:500 Pab9.3 anti-HER-2 polyclonal antiserum (Berlex Biosciences), 2 μg/ml 2F12 anti-HER-3 monoclonal antibody (Neomarkers), 1:500 anti-p85 polyclonal antiserum (Upstate Biotechnology), or 1:500 anti-SHC polyclonal antiserum (Transduction Laboratories) in TTBS plus 3% milk at room temperature for 60 min with moderate agitation. The blots were then rinsed in TTBS three times (5 min each) with moderate agitation, incubated with biotinylated antimouse IgG or biotinylated antirabbit IgG secondary antibody (Vector Laboratories) at a 1:750 dilution in TTBS at room temperature for 60 min with moderate agitation, rinsed in TTBS three times for 5 min each with moderate agitation, and then incubated with ABC streptavidin HRP reagents (Vector Laboratories) diluted in TTBS at room temperature for 60 min with moderate agitation. After the final rinsing of the blots in TTBS (three times; 5 min each) with moderate agitation, the bands were visualized with enhanced chemiluminescent substrate (Pierce) according to the manufacturer's instructions. Negatives exposed by chemiluminescent substrate were scanned and quantified using the IQ25 Intelligent Quantifier system (Bio Image).

**Cell Growth Assays.** For the monolayer growth assay, the cell lines were plated in 6-well tissue culture plates at a density of $10^5$ cells/well in medium containing all of the supplements listed above minus the insulin and EGF. After 24 h, the medium was replaced with SF medium without growth factors, medium with 5 μg/ml insulin and 10 ng/ml EGF, or medium with 10 ng/ml HRG-β, and the media were changed every other day. Cell counts were taken after 24 h to measure the plating efficiency and at day 10 to measure the proliferation during 9 days in SF culture. For counting cells, the cells from triplicate wells for each condition were trypsinized and counted using a hemocytometer. For the soft agarose assays, the cells were plated in 24-well plates within 0.3% agarose at a density of $2.5 \times 10^4$cells/0.25 ml atop a 0.25-ml layer of 0.6% agarose in growth medium with or without 10 ng/ml HRG-β and cultured for a month before counting colonies of at least 50 μm in diameter.

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